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2. Li, P., Yu, X., Ge, K., Melamed, J., Roeder, R.G. and Wang, Z. (2002) Heterogeneous Expression and Functions of Androgen Receptor Coactivators in Primary Prostate Cancer. *J. Am. Path.* **161**:1467-1474.
3. Liu, G.Z., Wang, H. and Wang, Z. (2003). Identification of a Highly Conserved Domain in the AndrogenReceptor That Suppresses the DNA-Binding Domain-DNA Interactions. *J. Biol. Chem.*, **278**:14956-60.
4. Hosohata, K., Li, P., Hosohata, Y., Qin, J., Roeder, R.G. and Wang, Z. (2003). Purification and identification of a Novel Complex Which Is Involved in Androgen Receptor-Dependent Transcription. *Mol. Cell. Biol.*, **23**:7019-7029.
5. Gao, S., Liu, G.Z. and Wang, Z. (2004). Modulation of Androgen Receptor-Dependent Transcription by Resveratrol and Genistein in Prostate Cancer Cells. *The Prostate*, **59**:214-225.

## INTRODUCTION

The androgen receptor mediates the biological functions of androgens in gene expression and is implicated in prostate cancer. Androgen ablation therapy, while effective in early androgen-dependent stages, nonetheless fails in the androgen-independent stages of advanced prostate cancer. Although the mechanism for the clinical response to androgen withdrawal therapy is not clear, androgen-independent progression has been associated with mutations or amplification of the androgen receptor gene and activation of intracellular signal transduction pathways that stimulate the androgen receptor function. Insights into this problem and a possible therapy for prostate cancer may be gained from a detailed understanding of the molecular mechanisms by which androgen receptor activates key target genes in malignant prostate cells. Our hypothesis is (i) that the transition from androgen-dependent to androgen-independent prostatic cancer involves alterations in the levels or activities of coactivators that interact physically and/or functionally with AR to activate genes whose expression contributes to malignancy and (ii) that an understanding of the nature, regulation and mechanism of action of these factors can facilitate the development of anti-prostatic cancer therapy. The general objective is to use completely defined cell-free transcription systems both to identify novel AR-associated cofactors, isolated by conventional fractionation and/or affinity methods, and to investigate the mechanism of action of these and previously identified candidate coactivators. Toward this objective we will (i) investigate purified androgen receptor and cognate cofactor functions in cell-free transcription systems reconstituted with general initiation factors and cofactors, wild type and mutant androgen receptors, and both DNA and chromatin templates, (ii) identify by complementation assays, purify by affinity methods, and characterize mechanistically additional (co)factors that act in association with androgen receptor on androgen receptor-activated genes and (iii) investigate possible changes either in the levels or in the activities (functional modifications) of these factors (e.g. in response to other signal transduction pathways) during prostate cancer development. This approach might provide new drug development insights into targeting the androgen receptor pathway downstream of the point of ligand-receptor interaction.



## **BODY**

- Task 1 To analyze the cofactor requirements and mechanisms of action of AR on purified DNA templates in a purified system (months 1-12):**
- a. **express and purify various cofactors (months 1-6).**
  - b. **immunopurify cofactor complexes from cell lines (months 1-6).**
  - c. **perform transcription assays (months 1-6).**

We have fully finished the proposed research in this task and the obtained results have been published in two papers (Appendices I and IV). We have established a well-defined cell-free transcription system comprised of the recombinant TFIIA, TFIIB, TFIIE, TFIIF, PC4 and the natural immuno-purified TFIID, TFIIH and RNA Pol II complexes (Appendix I). Using a synthetic ARE-containing DNA template, we have systematically analyzed requirements for the TRAP/Mediator components, SRC1, p300 and miscellaneous coactivators. Amino-terminal enhancer of split (AES) strongly inhibited AR-dependent transcription both in vitro and in vivo (Appendix I) and the p44-containing complex enhanced AR-dependent transcription in vitro (Appendix IV). Thus, we have identified new negative and positive regulatory pathways for AR through an associated co-repressor or coactivator. SRC1, p300, ARA70 and PGC-1 failed to activate AR-dependent transcription in this reconstituted system (First Annual Report, Figure 1). In contrast, the immuno-purified Mediator complex enhanced AR function and protein-protein pull-down assays revealed that that AR directly interacts with TRAP220 and with TRAP80 (First Annual Report, Figures 1 and 2). Consistently with these in vitro observations, transfection assays with TRAP220<sup>-/-</sup> mouse embryo fibroblasts (derived from embryonic knockout mice) (3) also showed that TRAP220 is essential for optimal activation by AR lacking the LDB but (under these suboptimal assay conditions in non-prostate cells) not for activation by intact AR (First Annual Report, Figure 3). These results thus implicate TRAP220 in a ligand-independent activation function of AR and are of potential significance with regard to androgen-independent growth of prostate cancer cells.

- Task 2 To analyze the cofactor requirements and mechanisms of action of AR on chromatin templates (months 1-12):**
- a. **purify human core histones from HeLa cells (months 1-6)**
  - b. **express and purify NAP1, ACF1 and ISWI (months 1-6)**
  - c. **assemble chromatin in vitro (months 6-12)**
  - d. **perform transcription assays on the chromatin template (months 6-12)**

Toward this goal for AR, we have established a chromatin assembly system based on purified components (First Annual Report, Figure 4). In this system we observed the p300 and acetyl-CoA dependent transcription from the chromatin template using the GAL4-VP16 as a activator (First Annual Report, Figure 4). However, the AR-dependent transcription from the chromatin template was not observed even in the presence of SRC1, p300, acetyl-CoA, and the androgen (R1881) (Second Annual Report, Figure 1). Therefore, the AR-driven transcription with the chromatin template did not work out as expected. One possible reason for the failure to obtain AR-dependent transcription from



the chromatin template is that additional cofactors required for AR-dependent transcription from the chromatin template are lacking in our cell free system. The future work will be to include various cofactors such as the Mediator (2), the p44-containing complex (Appendix IV), and the ATP-dependent chromatin-remodeling complex (SWI/SNF complex) (4) in the cell-free transcription system with the chromatin template in the future.

**Task 3      To determine activators and cognate cofactors acting in conjunction with AR on natural promoters (months 1-12):**

- a. analyze the Probasin promoter elements (months 1-6)
- b. express and purify the involved activators (months 6-12)
- c. perform transcription assays using the recombinant activators (months 6-12)

In order to study AR function in the context of natural promoters and synergistic interactions with other activators. We have determined the promoter elements on the probasin promoter and found that the Myb-binding site is a critical element. The recombinant Myb was expressed in bacteria and purified through Ni-NTA agarose column. In the purified reconstituted transcription system containing the purified general factors, cognate coactivator (PC4), and purified AR, we did not observed the synergism between AR and Myb. Chromatin templates may be necessary to elicit synergism. Due to the failure to obtain the AR-dependent transcription from the chromatin template, this research was not pursued further.

**Task 4      To establish the nontumor prostate and androgen-independent prostate cancer cell lines that stably expresse a FLAG-tagged androgen receptor (months 13-24):**

- a. transfect the cell lines MDA PCa 2b and RWPE-1 with pBabe-neo-f:AR (months 13-18)
- b. select the drug resistant cell line (months 13-18)
- c. analyze the drug resistant cell lines (months 19-24)

BPH (non-neoplastic) and PC3 (androgen-independent tumorigenic) prostate cell lines are widely used and are available from the American Type Cell Culture (ATCC) (5). They grow well in the RPI1640 medium in tissue culture. In contrast, RWPE-1 (non-neoplastic) and MDA PCa2b (androgen-independent tumorigenic) are not commonly used and need special medium for their growth in the tissue culture (1, 6). Therefore, we have chosen BPH and PC3 cells instead of RWPE-1 and MDA PCa2b cells for the proposed studies. BPH and PC3 cell lines were transfected with pBabe-Neo-FLAG:AR and selected with G418. The G418 resistant cell lines were analyzed for the expression of the FLAG-tagged AR by Western blot analysis with anti-AR antibody. Two PC3 cell lines (PC3-AR-2 and PC3-AR-9) express the high levels of AR (Second Annual Report, Figure 2). Consistent with the published observations (7), we found that the PC3 cells expressing ectopic AR grow slower and have the higher apoptotic rate than the parent



PC3 cells. At the same time and under the same conditions, we did not obtain any BPH cell line expressing the ectopic FLAG-tagged AR. This indicated that AR might inhibit the growth of BPH cells. This is consistent with our recent observations that the expression of AR in the primary prostate epithelial cells was lost after growing in the tissue culture for two weeks. Currently, we are using the Tet-inducible system (Clontech) to express the FLAG-tagged AR in BPH cells. In the future we will immunopurify the AR-containing complexes from cytoplasmic or nuclear extracts made from these cell lines growing in the presence or in the absence of the synthetic androgen R1881.

**Task 5**     *Purification and peptide sequence analysis of f:AR-associated proteins (months 13-24):*

- a. *grow f:AR-LNCaP cell line (months 13-24)*
- b. *make nuclear and cytoplasmic extracts from f:AR-LNCaP cells (months 13-24)*
- c. *analyze sequences of the f:AR-associated polypeptides (months 18-24)*

This task has been fully completed. The results have been published (Appendix IV).

**Task 6**     *Cognate cDNA cloning, recombinant protein expression and antibody production (months 25-36):*

- a. *clone cognate cDNA of the f:AR-associated polypeptides (months 25-30)*
- b. *express and purify the f:AR-associated polypeptides (months 25-30)*
- c. *make polyclonal antibodies against the f:AR-associated polypeptides (months 25-30)*

This task has been fully completed. The results have been published (Appendix IV).

**Task 7**     *Functional analysis of f:AR-associated proteins (months 30-36):*

- a. *perform in vitro transcription assays with the f:AR-associated polypeptides (months 30-36)*
- b. *perform western blotting analysis of the f:AR-associated polypeptides (months 30-36)*
- c. *perform in vivo transient transfection analysis with the f:AR-associated polypeptides (months 30-36)*

This task has been fully finished. The results have been published (Appendix IV).

**Task 8**     *Cell specificity and function of fAR-associated cofactors (months 30-36):*

- a. *perform Western blotting analysis (months 30-36)*
- b. *perform in situ hybridization with prostate cancer samples (months 30-36)*

This task has been fully completed. The results have been published in two papers (Appendices II and IV).



## KER RESEARCH ACCOMPLISHMENTS

1. Established a cell-free transcription system to investigate functions and mechanisms of purified androgen receptor and cognate cofactors.
2. Purification, identification and cloning of novel cofactors (AES and p44) that regulated AR-dependent transcription both *in vitro* and *in vivo*.
3. Found that change of expression levels of AR cofactors may play important roles in prostate growth and tumorigenesis.

## REPORTABLE OUTCOMES (PUBLICATIONS)

1. Yu, X., Li, P., Roeder, R.G. and Wang, Z. (2001). Inhibition of androgen receptor-mediated transcription by the amino-terminal enhancer of split, a Groucho/TLE family protein. *Mol. Cell. Biol.* **21**:4614-4625.
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## CONCLUSIONS

We have fulfilled the proposed tasks. We have investigate AR and cognate cofactor functions in cell-free transcription systems reconstituted with general initiation factors, cofactors, androgen receptors, and androgen-responsive templates. In this system, we observed an AR-dependent transcription, which could be positively or negatively modulated by various AR cofactors. Relevant to the mechanism involved, we have identified two Mediator subunits as potential targets for AR and TFIIE as a potential target for the negative AR cofactor (AES). We have identified AES and p44 as new AR-interacting proteins, which positively or negatively modulate AR transactivation. We also investigate the expression of AR and 10 cofactors in primary prostate cancers and found near constant expression of AR and heterogeneous expression of AR cofactors. Modulation of cofactor expression affected the proliferation and colony formation of prostate tumor cells. Together, these findings

indicate that the change of expression levels of AR cofactors may play important roles in prostate growth and tumorigenesis.

#### **PERSONNEL RECEIVING PAY FROM THE REASERCH EFFORT**

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#### **REFERENCES**

1. **Bello, D., M. M. Webber, H. K. Kleinman, D. D. Wartinger, and J. S. Rhim.** 1997. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. *Carcinogenesis* **18**:1215-23.
2. **Ito, M., and R. G. Roeder.** 2001. The TRAP/SMCC/Mediator complex and thyroid hormone receptor function. *Trends Endocrinol Metab* **12**:127-34.
3. **Ito, M., C. X. Yuan, H. J. Okano, R. B. Darnell, and R. G. Roeder.** 2000. Involvement of the TRAP220 component of the TRAP/SMCC coactivator complex in embryonic development and thyroid hormone action. *Mol Cell* **5**:683-93.
4. **Marshall, T. W., K. A. Link, C. E. Petre-Draviam, and K. E. Knudsen.** 2003. Differential requirement of SWI/SNF for androgen receptor activity. *J Biol Chem.*
5. **Mitchell, S., P. Abel, M. Ware, G. Stamp, and E. Lalani.** 2000. Phenotypic and genotypic characterization of commonly used human prostatic cell lines. *BJU Int* **85**:932-44.
6. **Navone, N. M., M. Olive, M. Ozen, R. Davis, P. Troncoso, S. M. Tu, D. Johnston, A. Pollack, S. Pathak, A. C. von Eschenbach, and C. J. Logothetis.** 1997. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin Cancer Res* **3**:2493-500.
7. **Yuan, S., J. Trachtenberg, G. B. Mills, T. J. Brown, F. Xu, and A. Keating.** 1993. Androgen-induced inhibition of cell proliferation in an androgen-insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA. *Cancer Res* **53**:1304-11.



## Inhibition of Androgen Receptor-Mediated Transcription by Amino-Terminal Enhancer of split

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**A yeast two-hybrid assay has identified an androgen-dependent interaction of androgen receptor (AR) with amino-terminal enhancer of split (AES), a member of the highly conserved Groucho/TLE family of corepressors. Full-length AR, as well as the N-terminal fragment of AR, showed direct interactions with AES in *in vitro* protein-protein interaction assays. AES specifically inhibited AR-mediated transcription in a well-defined cell-free transcription system and interacted specifically with the basal transcription factor (TFIIE) in HeLa nuclear extract. These observations implicate AES as a selective repressor of ligand-dependent AR-mediated transcription that acts by directly interacting with AR and by targeting the basal transcription machinery.**

Androgen receptor (AR) is a member of the superfamily of ligand-inducible transcription factors and mediates the biological actions of androgens (19). Like other superfamily members, AR contains a central DNA-binding domain, a C-terminal ligand-binding domain with an associated AF-2 activation domain, and a large N-terminal region containing the AF-1 activation domain (4, 26). Nuclear receptors regulate the transcription of their target genes through the agency of various coactivators and corepressors that are recruited to target genes through interactions with promoter-bound receptors (56). Many of the known coactivators for nuclear receptors contain histone acetyltransferase activities and are thought to act mainly through targeted chromatin structural perturbations that facilitate the subsequent recruitment (to the promoter) and function of other transcriptional coactivators and basal transcriptional factors (3). Transcriptional corepressors, by contrast, mediate repression by various nuclear receptors. Some nuclear receptors (including retinoid receptor, thyroid hormone receptor, vitamin D receptor, and certain orphan receptors) that are not associated with heat shock proteins in their unliganded state repress transcription by recruitment of corepressor complexes (15, 35). Corepressor complexes contain histone deacetylase (HDAC) activities that maintain chromatin in a configuration that excludes functional interactions of the general transcriptional machinery with the promoter. In contrast, unliganded steroid receptors (including AR) generally associate with heat shock proteins and, upon ligand binding, dissociate from the heat shock proteins, translocate to the nucleus, and associate with coactivators to activate or repress target genes (30).

Another type of corepressor, implicated in the function of other types of repressors, is the Groucho/TLE family (see Fig. 1D) (5, 11). The larger family members such as *Drosophila* Groucho and its mammalian homologues, the TLE proteins (transducin-like enhancer of split [TLE1-3]), share five do-

main. A carboxyl-terminal WD-40 repeat domain (WD-40) and an amino-terminal glutamine-rich domain (Q) are highly conserved. In the much less well-conserved central region, there is a loosely conserved CcN motif (CcN), consisting of putative cdc2 kinase and casein kinase II phosphorylation sites, and two poorly conserved regions (GP and SP) that are characteristically rich in either glycine and proline (GP) or serine and proline (SP) residues. A shorter family member, human TLE4, is similar except for the absence of the amino-terminal Q and GP domains. The shortest family member, amino-terminal enhancer of split (AES), shares only the first two regions of the amino terminus.

The Q domain mediates both homo- and hetero-oligomerization between Groucho/TLE family proteins, whereas the WD-40 repeats appear to mediate protein-protein interactions with relevant DNA-binding activators and repressors. Groucho/TLE proteins do not have recognizable DNA-binding domains but can repress transcription directly if tethered to DNA through a Gal4 DNA-binding domain or if recruited to DNA through interactions with other DNA-binding activators and repressors. The function of AES remains controversial. It was suggested that AES might act as an inhibitor of Groucho/TLE corepressors by dominant negative mechanisms (28, 45). On the other hand, AES has been shown to mediate Blimp-1-dependent repression of the beta interferon gene (41) and to repress NF- $\kappa$ B-driven gene expression (51) *in vivo*.

Here we demonstrate that AES physically interacts with human AR both *in vivo* and *in vitro* and that it represses AR-dependent transcription both in transient-transfection assays and in a purified cell-free transcription system. In addition, we find that AES interacts selectively with the basal transcription factor TFIIE. These observations indicate that AES represses AR-driven transcription by directly targeting the basal transcription machinery.

### MATERIALS AND METHODS

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**Yeast screening.** The yeast two-hybrid screening was performed as previously described with minor modifications (57). Briefly, an expression plasmid encoding the Cyto-trap bait was generated by inserting the cDNA sequences of human AR into pSos, a yeast shuttle vector. *Saccharomyces cerevisiae* strain cdc25H was



transformed sequentially with pSos-AR and human prostate cDNA library expression plasmids (Stratagene). The positive clones were those that grow on the plates with galactose and 100 nM R1881 at 37°C but not on galactose plates in the absence of R1881. Plasmids were rescued from each of these positive colonies and identified by nucleotide sequencing.

**Mammalian two-hybrid analysis.** Expression vectors that encode hybrid polypeptides were produced by inserting AES cDNA sequences into the pCMV-GAL4 vector or by inserting AR cDNA sequences into the pVP-FLAG7 vector (57). A mammalian two-hybrid assay was conducted in 293T cells as described previously (52), except that when indicated, transfected cells were incubated for 40 h with medium containing 100 nM R1881. The pRL-LUC plasmid was included in each culture of transfected cells as an internal control. The luciferase activity was determined using the Dual-luciferase assay system (Promega).

**Transient transfection.** The AR and AES expression vectors for transfection assays were constructed by inserting their corresponding cDNA sequences into pcDNA3.1. The AR-responsive reporter gene ARE4-LUC contains four AR-responsive elements ahead of the E4 basal promoter and the luciferase gene. HeLa Z cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Transfections were performed using SuperFect reagent (Qiagen). Briefly,  $10^5$  cells were plated onto 24-well plates approximately 24 h before transfection. After the plates were washed with phosphate-buffered saline, cells in each well were transfected with 50 ng of an expression vector (AR, estrogen receptor [ER], or thyroid hormone receptor [TR]), 100 ng of the reporter plasmid, 5 ng of the pRL-LUC internal control plasmid, and the indicated amount of the AES expression vector. The total amount of DNA was adjusted to 1 µg with pcDNA3.1. Transfections were conducted in phenol red-free RPMI 1640 medium, and 2 h later the medium was changed either to phenol red-free RPMI 1640 medium plus 10% charcoal dextran-stripped fetal bovine serum or to regular medium containing 100 nM R1881, 1 µM β-estradiol, or 10 nM T3. The cells were cultured for another 48 h and harvested for luciferase assays (Promega). For trichostatin A (TSA) treatment, 10 ng of TSA per ml was added to transfected cells 24 h before harvest. Three independent experiments were carried out in each case for statistical analysis.

**Purification of transcription factors.** Histidine-tagged TFIIAα and TFIIAγ were expressed in bacteria via the pRSET vector and purified on Ni-nitrilotriacetic acid (NTA)-agarose in the presence of 6 M urea (8). TFIIAα was reconstituted with a combination of equimolar amounts of purified TFIIAα and TFIIAγ and dialyzed against BC300–0.1% NP-40. The FLAG-tagged TFIIAβ was expressed via vector pET15d and purified on M2 agarose. TFIIA was reconstituted with a combination of equal amounts of TFIIAα and TFIIAβ. Bacterially expressed histidine-tagged TFIIβ was purified on Ni-NTA-agarose and phosphocellulose. Histidine-tagged TFIIα and FLAG-tagged TFIIβ were expressed in bacteria and purified on Ni-NTA-agarose and M2 agarose, respectively, and TFIIα was reconstituted with a combination of two subunits and further purified through M2 agarose. TFIIβ was expressed and reconstituted as reported previously (53). Bacterially expressed untagged PC4 was purified through heparin-Sepharose and phosphocellulose (13). Histidine-tagged GAL4-VP16 was expressed in bacteria and purified through Ni-NTA-agarose and S-Sepharose.

Nuclear extract was made from the FLAG-tagged TAF135 cell line (31) and further fractionated by phosphocellulose and DEAE cellulose (DE52) chromatography. FLAG-tagged TFIIID (f:TFIID) was isolated from the 0.3 M KCl fraction of a DE52 column by M2 agarose affinity purification. HeLa cell lines stably expressing FLAG-tagged RPB9 and FLAG-tagged XRB1 were established, and FLAG-tagged RNA polymerase II (f:PolII) and TFIIH (f:TFIIH) were purified from these cell lines by described procedures (54). Recombinant human androgen receptor was expressed in Sf9 cells via a baculovirus vector as a FLAG-tagged fusion protein and purified on M2 agarose.

**In vitro transcription and primer extension.** To create the template pARE-E4, a DNA fragment containing four copies of the androgen-responsive element (AGAACAGCAAGTGCT) from the PSA promoter was inserted into *Sph*I and *Xba*I sites of the vector pG5E4. Transcription reactions were carried out in a final volume of 25 µl, and the reaction mixtures contained 90 fmol of supercoiled plasmid DNA template, 20 mM HEPES (pH 7.9), 12% glycerol, 6 mM MgCl<sub>2</sub>, 70 mM KCl, 5 mM dithiothreitol (DTT), 600 µM each ATP, UTP, CTP, and GTP, 40 U of recombinant RNasin, 0.5 mg of bovine serum albumin per ml, 12 ng of TFIIA, 30 ng of TFIIβ, 2 µl of f:TFIID, 0.5 µl of f:TFIIH, 12 ng of TFIIβ, 6 ng of TFIIα, 150 ng of PC4, 1 µl of f:PolII, 30 ng of human AR, and various amounts of different cofactors. After a 60-min incubation at 30°C, the transcription reactions were stopped by adding 175 µl of stop solution (1% sodium dodecyl sulfate, 5 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 20 µg of glycogen, 40 µg of proteinase K) and incubating the mixture for 20 min at 37°C. RNA was extracted with phenol-chloroform and precipitated with ethanol.

It was then hybridized with the kinase <sup>32</sup>P-labeled primer CGCCAAGCTATT TAGGTGACACTAT (5' end labeled;  $1 \times 10^6$  to  $2 \times 10^6$  cpm) in 20 µl of hybridization buffer (10 mM Tris-HCl [pH 7.5], 250 mM KCl, 1 mM EDTA) for 90 min at 37°C. The primer extension reaction was started by adding 40 µl of extension reaction solution (75 mM Tris-HCl [pH 8.0], 15 mM DTT, 12 mM MgCl<sub>2</sub>, 75 µg of actinomycin D per ml, 12 U of recombinant RNasin, 750 µM each dATP, dTTP, dCTP, and dGTP, 100 U of SuperScript RNase H reverse transcriptase), and the reaction mixture was incubated for 90 min at 37°C. The cDNA products were extracted with phenol-chloroform; precipitated with ethanol; dissolved, and denatured (100°C for 3 min) in 10 µl of 95% formamide containing 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF; and finally analyzed on a 6% polyacrylamide–7 M urea gel.

**Protein-protein interaction assay.** Recombinant glutathione S-transferase (GST) fusion (expressed in bacterial cells) or FLAG-tagged (expressed in insect cells) proteins (1 µg) were immobilized on 10 µl of glutathione or M2 agarose beads, respectively. Then 10 µl of beads was incubated for 2 h at 4°C with 5 µl of rabbit reticulocyte lysate containing [<sup>35</sup>S]Met-labeled proteins or 100 µl of HeLa nuclear extract (60 µg proteins) in a final volume of 200 µl containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 150 mM KCl, 0.1% NP-40 and 0.5 mg of BSA per ml. The beads were washed five times (1 ml each) with the incubation buffer, boiled in 10 µl of the 2× SDS gel sample buffer, and analyzed by autoradiography or Western blot analysis. For the coimmunoprecipitation assay, 10 µl of M2 agarose beads was incubated with 250 µl of whole-cell extract from transfected 293T cells in BC150–0.1% NP-40 for 2 h. The beads were washed with the incubation buffer and analyzed by Western blotting.

**In situ hybridization.** The archival normal prostate tissues were obtained during radical prostatectomy of prostate cancer patients at New York University Medical Center under an Institutional Review Board-approved protocol. The procedure for in situ hybridization was as described previously (29). Briefly, the sections (4 µm) of prostate tissues were hydrated, postfixed in 4% paraformaldehyde, treated with proteinase K, and deacetylated. The prehybridization and hybridization were performed at 68°C. The 536-bp AR (nucleotides 2224 to 2716) and the 648-bp AES (nucleotides 353 to 957) cDNA fragments containing T7 and T3 promoters at each end was generated by PCR. The <sup>33</sup>P-labeled probe RNAs (sense and antisense) were generated by in vitro transcription with T7 and T3 RNA polymerases, respectively, and hybridized to the slides containing prostate tissue specimens. After being washed, the slides were exposed for 2 to 3 weeks and then counterstained with hematoxylin and eosin.

## RESULTS

**N-terminal domain of AR interacts with the Groucho/TLE family protein AES.** Various coactivators and corepressors have been shown to play a critical role in mediating the functions of nuclear receptors (56). Although a number of AR-interacting coactivators have been identified (reviewed in references 4 and 19), we have used a yeast two-hybrid screening method to search for additional AR-interacting proteins. For this assay, full-length human AR (residues 2 to 919) was fused to human Sos (hSos) as a bait (Fig. 1A). The temperature-sensitive mutant *S. cerevisiae* strain cdc25H, which contains a point mutation in the yeast homolog (*cdc25*) of the hSos gene, cannot grow at 37°C but can grow at the permissive temperature (25°C). This yeast strain was used to screen a human prostate cDNA expression library fused to the v-Src myristylation sequence, which anchors the fusion protein to the plasma membrane. If the bait and target proteins physically interact, the hSos protein is recruited to the membrane, thereby activating the Ras signaling pathway and allowing the cdc25H yeast strain to grow at 37°C.

Approximately 2 million transformants from the prostate cDNA library were screened, and 35 positive clones were obtained. Nucleotide sequence determination and comparison with GenBank databases (National Center for Biotechnology Information) revealed seven clones that encoded the human AES (5, 11). To confirm that the interactions between AR and AES are specific, human AES was fused to the Gal4 DNA-



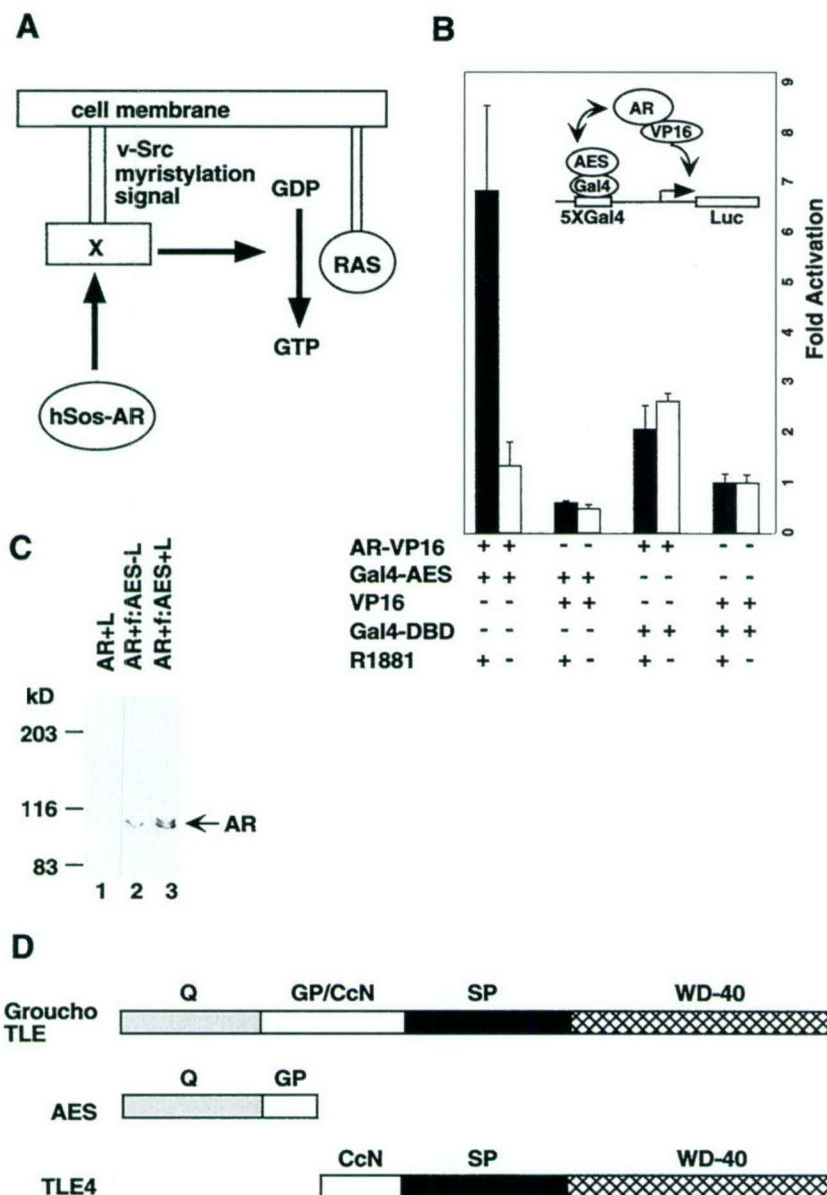


FIG. 1. AR interacts with AES in vivo. (A) Schematic diagram of the Ras signaling pathway utilized in the yeast two-hybrid system. (B) Mammalian two-hybrid assay with Gal4-AES and AR-VP16 fusion proteins in 293T cells. 293T cells were cotransfected with 1  $\mu$ g of either AR-VP16, Gal4-AES, VP16, or Gal4-DBD in the presence or absence of R1881 (100 nM), along with 100 ng of pG5-Luc reporter plasmid. A significant interaction was detected only between AR and AES. (C) AR was coimmunoprecipitated with AES. 293T cells were transfected with AR (lane 1) or AR and FLAG-tagged AES (lanes 2 and 3) in the presence (lanes 1 and 3) or absence (lane 2) of R1881 (100 nM). Whole-cell extracts were made from the transfected cells and incubated with M2 agarose beads. The immunoprecipitated proteins were analyzed by Western blotting with an anti-AR antibody. (D) Domain structures of three forms of the Groucho/TLE family proteins.

binding domain and AR was fused to the VP16 transcriptional activation domain. These constructs were transfected into 293T cells with a reporter containing five Gal4-binding sites and the E1b core promoter fused to the luciferase gene, and activation of luciferase reporter was measured in the absence and presence of ligand (R1881). A sevenfold activation of the reporter gene was observed in the presence of androgen but not in its absence, indicating that AR-AES interactions are hormone-dependent in vivo (Fig. 1B). As negative controls, neither coexpression of AR-VP16 with Gal4-DBD nor coex-

pression of Gal4-AES with VP16 resulted in significant ligand-dependent activation of the reporter (Fig. 1B). To further confirm the interaction of AR with AES in mammalian cells, we performed a coimmunoprecipitation using immobilized anti-FLAG monoclonal antibody (M2 agarose). AR was coimmunoprecipitated with AES from the whole-cell extract made from cells transfected with AR and FLAG-tagged AES in the presence of 100 nM ligand (R1881) (Fig. 1C, lane 3). In the absence of ligand, only trace amounts of AR were coimmunoprecipitated (lane 2). As a negative control, no AR was immu-

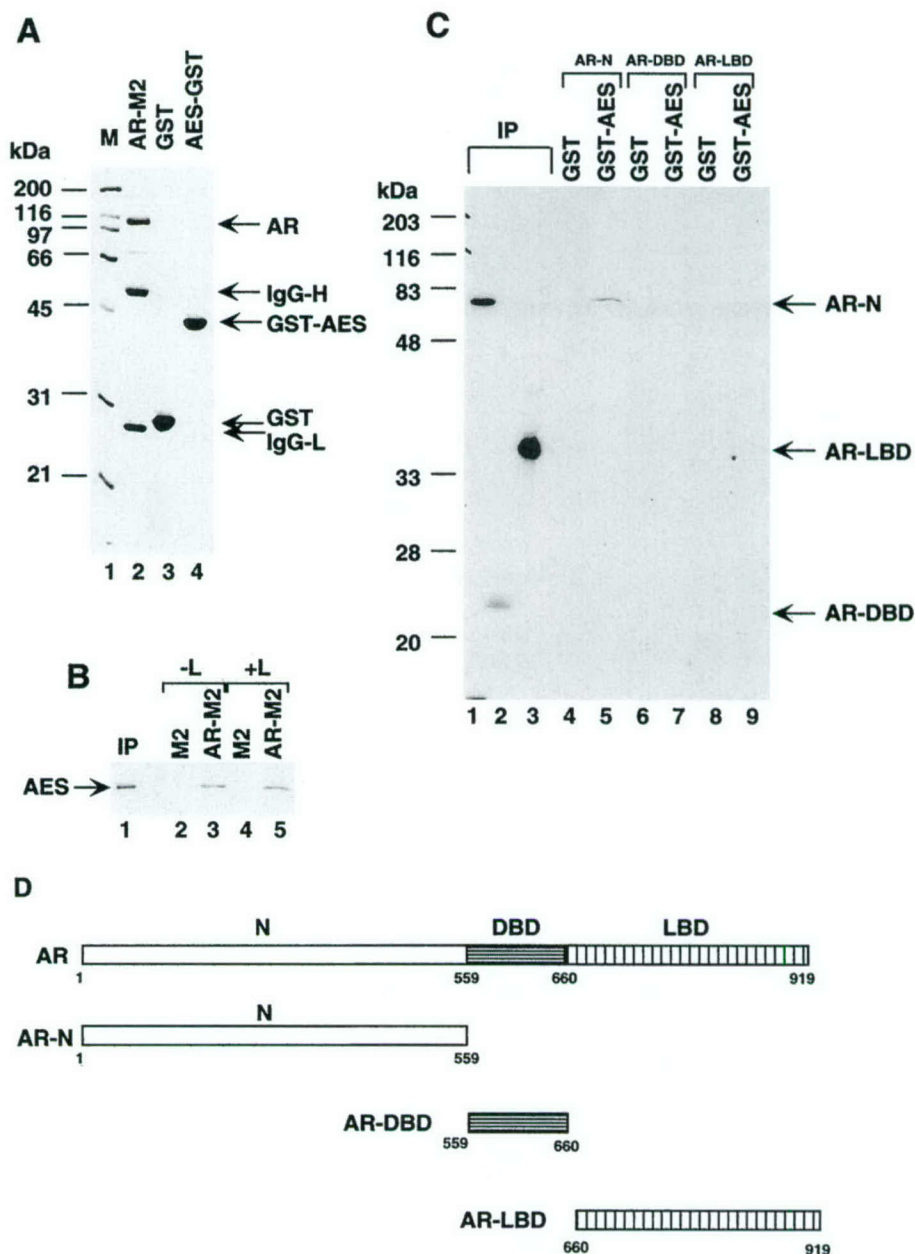


FIG. 2. The N-terminal part of AR directly interacts with AES in vitro. (A) SDS-PAGE (12% polyacrylamide) analysis of the M2 agarose-bound recombinant AR (lane 2), bacterially expressed and purified GST (lane 3), and GST-AES (lane 4) proteins. Standard molecular mass markers (M) (in kilodaltons) are shown in lane 1. IgG light (IgG-L) and heavy (IgG-H) chains of monoclonal antibody (M2) that dissociated from agarose beads by boiling with SDS sample buffer are indicated on the right. (B) AR interacts with AES in vitro independently of the ligand. Radiolabeled AES was incubated with M2 (lanes 2 and 4) or FLAG-tagged AR immobilized on M2 agarose beads (lanes 3 and 5) in the absence (lane 2 and 3) or presence (lanes 4 and 5) of 100 nM R1881. After the beads were washed, bound AES and 5% of the input (IP) (lane 1) were analyzed on by SDS-PAGE (12% polyacrylamide) and visualized by autoradiography. (C) The N-terminal part of AR is sufficient to bind to AES. GST (lanes 4, 6, and 8) or GST-AES (lanes 5, 7, and 9) proteins, immobilized on beads, were mixed with 5  $\mu$ l of in vitro labeled N-terminal (AR-N) (lanes 4 and 5), DNA-binding (AR-DBD) (lanes 6 and 7), and ligand-binding (AR LBD) (lanes 8 and 9) domains of AR. After the beads were washed, the bound proteins and 5% of the input (IP) (lanes 1 to 3) were analyzed by SDS-PAGE (12% polyacrylamide) and visualized by autoradiography. (D) Diagram of AR, AR DNA-binding domain, and AR ligand-binding domain.

noprecipitated by M2 agarose when the cell was transfected with AR alone (lane 1).

To further investigate the interactions of AES with AR, we performed in vitro protein-protein pull-down assays. In vitro-translated [ $^{35}$ S]AES was incubated with FLAG-tagged AR that

had been expressed in Sf9 cells and immobilized on M2 agarose beads (Fig. 2A, lane 2). Figure 2B shows that AES bound to AR-M2 (lanes 3 and 5) but not to unliganded M2 agarose beads (lanes 2 and 4). These interactions were found to be ligand independent (Fig. 2B, compare lane 5 with lane 3), a



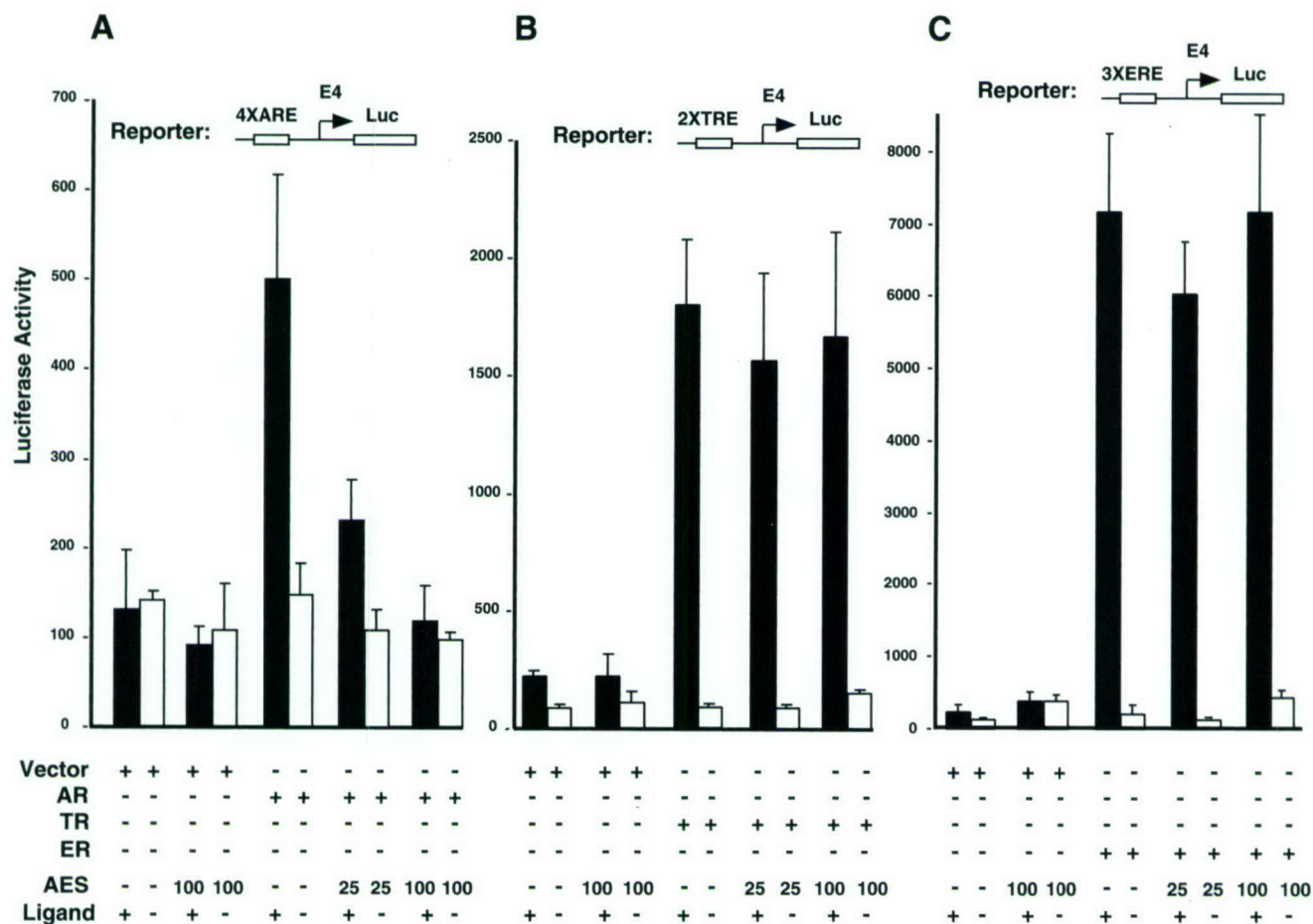


FIG. 3. AES inhibits AR-mediated transcription in vivo. (A) AES represses AR-dependent luciferase gene expression induced by AR in the presence of R1881. HeLa cells were transfected with 500 ng of 4×ARE-E4-luc reporter plasmid, 30 ng of pCMV-AR, and the indicated amounts of pCMV-AES expression plasmids. Cells were grown in the absence or presence of 100 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays. (B) TR-mediated T3-dependent luciferase gene expression is not suppressed by AES. The reporter construct contains 2×TRE, the E4 core promoter, and the luciferase gene. After transfection, HeLa cells were grown for 48 h in the absence or presence of 10 nM T3 before being harvested for luciferase assays. (C) ER-mediated estradiol-dependent luciferase gene expression is not suppressed by AES. The reporter construct contains 3×ERE, the E4 core promoter, and the luciferase gene. After transfection, HeLa cells were grown for 48 h in the absence or presence of 1  $\mu$ M estradiol before the luciferase assays were performed.

somewhat surprising observation in view of the observed ligand-dependent interactions in vivo (Fig. 1B). This discrepancy is probably because AR associates with heat shock proteins and other chaperones in vivo in the absence of androgen (30), thereby preventing its interactions with AES as well as other cofactors. To identify the AR domain that interacts with AES, the N-terminal, DNA-binding, and ligand-binding domains of AR (Fig. 2D) were expressed as  $^{35}$ S-labeled proteins and incubated with GST and GST-AES fusion protein immobilized on glutathione-agarose beads (Fig. 2A, lanes 3 and 4). As shown in Fig. 2C, the N-terminal part bound to GST-AES (lane 5) but not to GST alone (lane 4) whereas the DNA-binding and ligand-binding domains failed to interact (lanes 7 and 9). This demonstration that AES interacts with the AR N-terminal region is interesting in light of the significant role of this region in target gene activation by liganded AR (26).

**AES represses AR-dependent gene expression.** We then investigated the effect of AES on AR-dependent transcription by performing transient-transfection assays. The luciferase re-

porter plasmid containing four tandem copies of the PSA gene androgen-responsive elements (7) upstream of the minimal adenovirus E4 promoter (see Fig. 5C) was cotransfected with expression vectors for AR and/or AES into HeLa Z cells in the absence or presence of ligand (R1881). As shown in Fig. 3A, AR activated the reporter gene about fourfold in the presence of androgen, and coexpressed AES completely blocked this AR-dependent transactivation in a dose-dependent manner. In the absence of cotransfected AR or ligand (R1881), AES did not influence reporter gene activity, indicating that the inhibitory effect of AES on AR-dependent gene expression was not due to an effect on the E4 promoter. Similar results were obtained with the LNCaP prostate cancer cell line (data not shown).

To further examine whether the inhibitory effect of AES is specific for AR, we compared the effects of AES on the transcription of reporters containing the same E4 promoter under the control of TR and ER. As shown in Fig. 3B and C, TR and ER activated the reporter genes about 17- and 35-fold, respec-

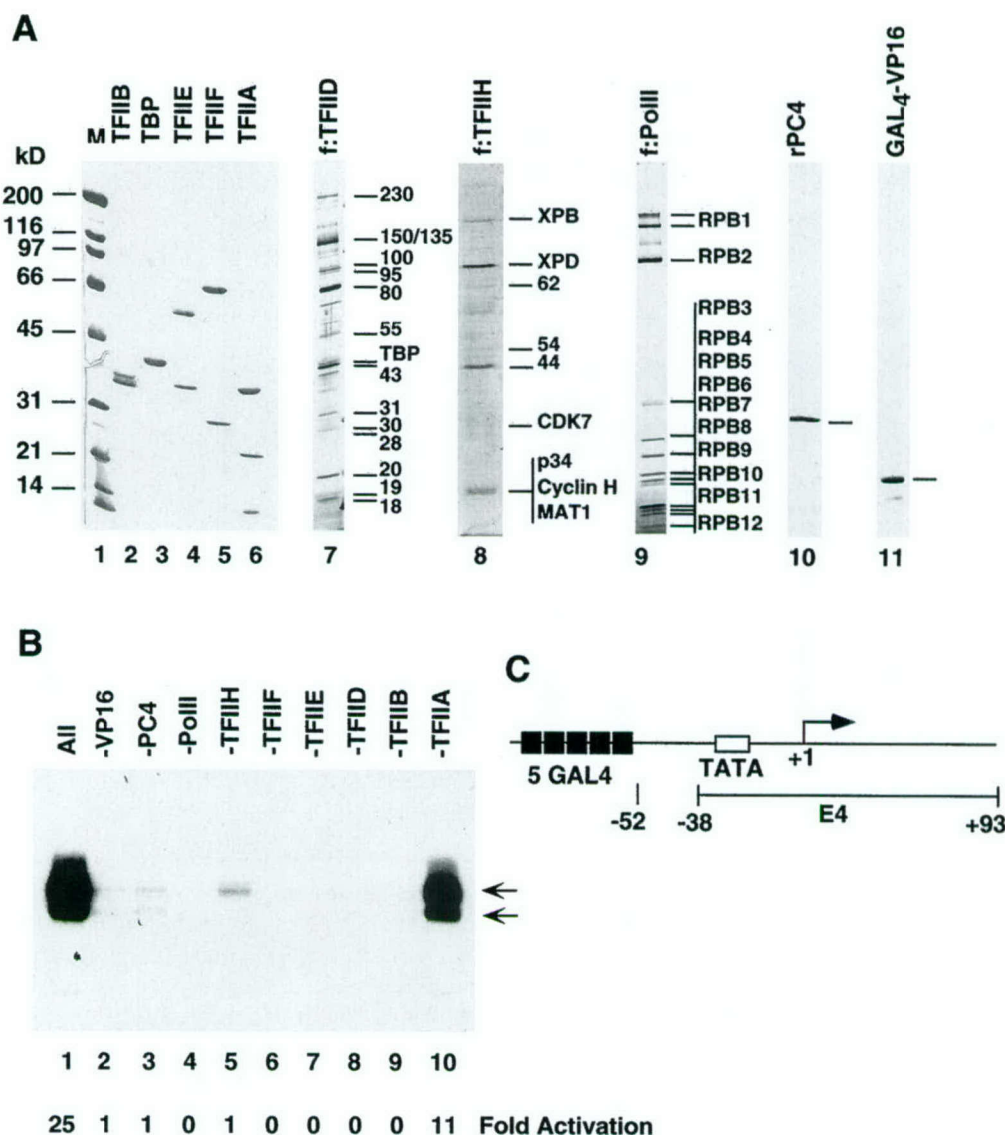


FIG. 4. Transcription activation by a model activator in a cell-free system reconstituted with purified factors. (A) SDS-PAGE analysis of purified factors. Coomassie blue R250 staining of purified recombinant activator GAL4-VP16 (lane 11), the general coactivator PC4 (lane 10), and the general initiation factors TFIIA, TFIIB, TFIIE, TFIIF, and TBP (lanes 2 to 6) was performed. Silver staining of the immunopurified FLAG-tagged multisubunit general initiation factors TFIID and TFIIH and RNA polymerase II (lanes 7 to 9) was performed. The subunits identified as integral subunits are indicated by size (in kilodaltons) or by name on the right. Some bands are difficult to visualize because of weak or negative staining. Unmarked bands represent either degradation products or contaminants that can be removed by further purification. Lane 1 shows molecular weight markers (M). (B) Activator-dependent transcription. Transcription was conducted with the purified components shown in panel A and the DNA template indicated in panel C. The two arrows show specifically initiated transcripts assayed by primer extension. A complete reaction with all factors is shown in lane 1, whereas reactions with single-factor omissions (indicated at the top) are shown in lanes 2 to 10. Fold activation above the basal level (-GAL4-VP16, lane 2) is indicated at the bottom. (C) Diagram of the model template. The template contains five tandem GAL4 sites adjacent to the adenovirus E4 core promoter.

tively, in the presence of their cognate ligands (T3 and estradiol). In contrast to its dramatic effect on AR-mediated transactivation, AES showed no effect on TR- or ER-mediated transcription. Western blot analysis revealed that the expression levels of ER were comparable to those of AR (data not shown). Hence, AES shows nuclear receptor-specific inhibitory effects *in vivo*.

**Establishment of a highly purified *in vitro* transcription system for activator function.** To study the mechanism of basal

and activator-dependent transcription, we established an activator-responsive complementation assay involving homogeneous recombinant and FLAG-tagged immunopurified natural general initiation factors (TFIIs) and positive cofactors (PCs) (43, 44). The recombinant factors expressed in and purified from bacteria included TFIIA (three subunits [Fig. 4A, lane 6]), TFIIB (one subunit [lane 2]), TFIIE (two subunits [lane 4]), TFIIF (two subunits [lane 5]), and PC4 (1 subunit [lane 10]). The multisubunit components purified from cell lines



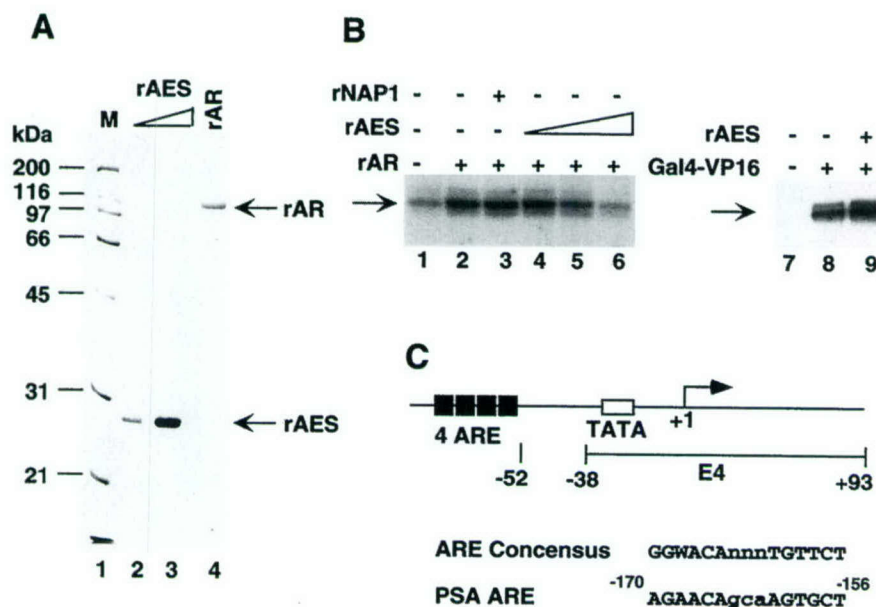


FIG. 5. AES represses AR-driven transcription in vitro. (A) SDS-PAGE analysis of recombinant AES and AR proteins. Portions of 50 (lane 2) and 200 (lane 3) ng of purified recombinant 6His-tagged AES expressed in bacteria and 100 ng of purified recombinant human AR (lane 4) expressed in Sf9 cells were subjected to SDS-PAGE with Coomassie blue R250 staining. (B) AES inhibition of AR-dependent transcription. A synthetic template containing four ARE elements (pARE-E4) was transcribed in a system reconstituted with the purified factors (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, Pol II, and PC4) as shown in Fig. 1, panel A, and 10 ng of affinity-purified (via f:Nut2) TRAP/Mediator complex (27). Other additions included 30 ng of rAR (lanes 2 to 6); 10 (lane 4), 30 (lane 5) or 100 (lanes 6 and 9) ng of rAES; 100 ng of rNAP1 (lane 3); and 30 ng of Gal4-VP16 (lanes 8 and 9). The specifically initiated transcript is indicated by an arrow and was monitored by primer extension. (C) Diagram of the synthetic ARE-containing promoter. The template (pARE-E4) contains four tandem copies of the ARE from the PSA promoter positioned upstream of the adenovirus E4 promoter.

expressing FLAG-tagged subunits included f:TFIID (~15 subunits [Fig. 4A, lane 7]), f:TFIIH (9 subunits [lane 8]), and f:Pol II (12 subunits [lane 9]). Recombinant GAL4-VP16 (lane 11) was used as an activator to establish the functionality of this particular assay system. The GAL4-VP16-responsive template pG5E4 (Fig. 4C) contains five Gal4-binding sites preceding the adenovirus E4 core promoter (from -38 to +93) (36). To determine whether all purified factors are required for transcription in our highly purified transcription system, we first tested a complete mixture of all GTFs, Pol II, Gal4-VP16, and PC4 with supercoiled DNA template (pG5E4) and then omitted individual factors. As shown in Fig. 4B, basal (activator-independent) transcription (lane 2) is completely dependent on TFIID (lane 8), TFIIB (lane 9), TFIIIE (lane 7), TFIIF (lane 6), and Pol II (lane 4) whereas activation (up to 25-fold) by GAL4-VP16 (lane 1) absolutely requires Pol II and all initiation factors other than TFIIA.

**AES represses AR-driven transcription in vitro.** For initial tests of AR function, we constructed a synthetic hybrid promoter (pARE-E4) containing four copies of an androgen response element (ARE) from the PSA promoter (7, 42) just upstream of the adenovirus E4 core promoter (Fig. 5C). This template was assayed in the above-described purified system supplemented with an affinity-purified TRAP complex previously shown to facilitate transcription by other nuclear receptors (12, 17, 58). In this system, the purified baculovirus-expressed recombinant AR (Fig. 5A, lane 4) activated transcription threefold (Fig. 4B, compare lane 2 with lane 1). To test its effect on AR-dependent transcription in this system,

human AES was expressed in and purified from bacteria (Fig. 5A, lanes 1 and 2). Addition of recombinant AES inhibited AR-dependent transcription in a dose-dependent manner and, at the highest level (100 ng), reduced it to the basal level (compare lane 6 and lane 1). As negative controls, 100 ng of recombinant mouse NAP1 expressed and purified in a manner identical to that for AES had no detectable effect on the AR-driven transcription (compare lane 3 and lane 2) and recombinant AES did not repress but slightly (1.5-fold) enhanced Gal4-VP16-driven transactivation (compare lane 9 and lane 8) in the same reconstituted system. These results suggest that AES specifically and directly represses AR-driven transcription in vitro.

**AES interacts with TFIIIE.** The structural resemblance of Groucho/TLE proteins to Tup1 (21), a general transcription repressor in yeast, suggested that the two proteins may function by a similar mechanism. More recently, Gromoller and Lehming (14) reported TUP1-mediated repression through physical interaction with the SRB7 subunit of the yeast Mediator complex. To test whether human AES uses a similar mechanism to repress transcription, we performed protein-protein pull-down assays. GST-AES failed to bind the human homolog (TRAP-Mediator complex, detected by anti-TRAP95 antibodies) of the yeast Mediator complex (27) in HeLa nuclear extract (Fig. 6A), as well as the independently expressed SRB7 and MED7 subunits of the human TRAP-Mediator complex (Fig. 6B, lanes 4 and 6). These results suggest that human AES, unlike yeast Tup1, may not directly interact with the Mediator complex.

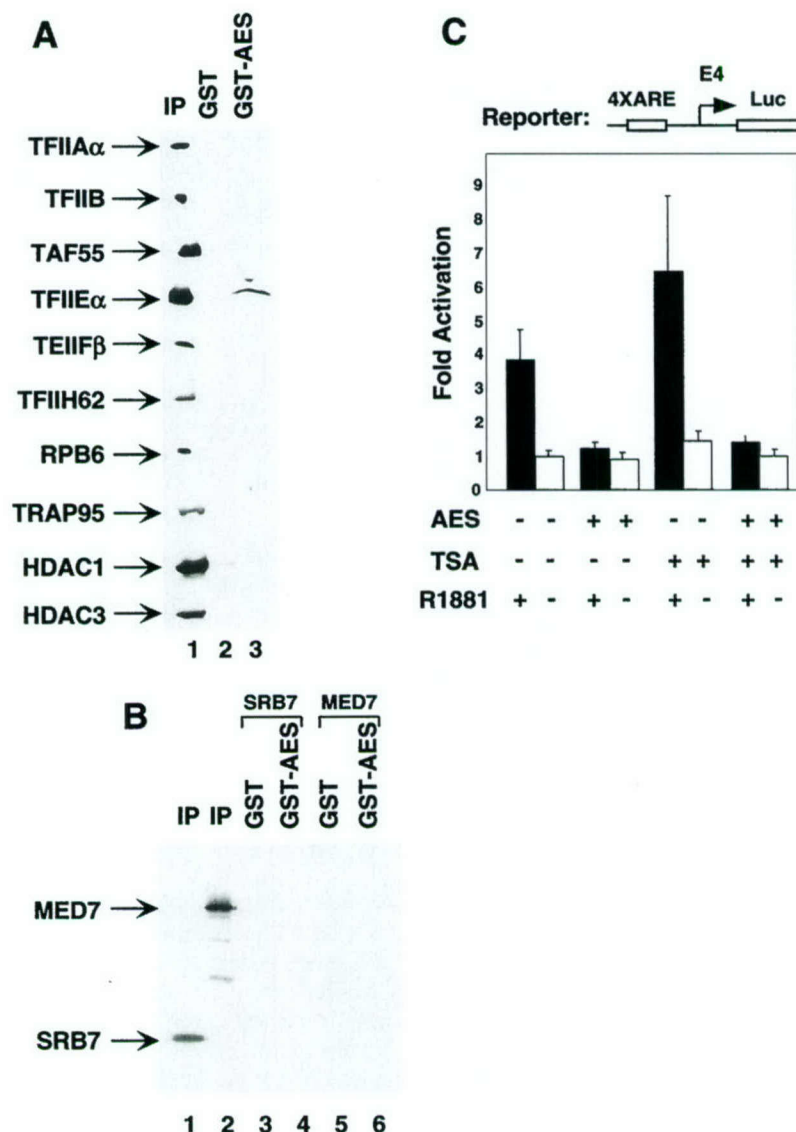


FIG. 6. AES interacts with TFIIE and represses AR-dependent transcription in the presence of TSA. (A) GST (lane 2) and GST-AES (lane 3), immobilized on agarose beads, were incubated with HeLa nuclear extract. The bound proteins (lanes 2 and 3) and 10% of the input (IP) were analyzed by Western blot assays with the corresponding antibodies indicated on the left. (B) AES does not interact with MED7 and SRB7. GST (lanes 3 and 5) or GST-AES (lanes 4 and 6) proteins, immobilized on beads, were mixed with 5  $\mu$ l of in vitro-labeled SRB7 (lanes 3 and 4) and MED7 (lanes 5 and 6). After the beads were washed, the bound proteins and 10% of the inputs (IP) (lanes 1 and 2) were analyzed by SDS-PAGE (15% polyacrylamide) and visualized by autoradiography. (C) AES inhibits AR-dependent transcription in the presence of TSA. HeLa cells were transfected with 100 ng of 4 $\times$ ARE-E4-luc reporter plasmid, 50 ng of pCMV-AR, and 100 ng of pCMV-AES expression plasmid. Cells were grown in the absence or presence of 100 nM R1881 and 10 ng of TSA per ml for 48 h after transfection and then were harvested for luciferase activity assays.

HDAC-containing complexes mediate the function of various corepressors in vivo (15, 35). To investigate whether AES also functions through these complexes, we performed transient-transfection assays in the presence of the general deacetylase inhibitor TSA. Figure 6C shows that AES still actively inhibits AR-dependent transcription in the presence of TSA and at a level similar to that observed in the absence of TSA. These results suggest that AES represses AR-dependent transcription by directly targeting the basal transcriptional machinery rather than through chromatin modifications involving recruitment of HDAC-containing corepressor complexes. This conclusion is further supported by the absence of demonstra-

ble interactions of AES with HDAC1- and HDAC3-containing complexes (16, 25) in HeLa nuclear extract (Fig. 6A). However, since the reporter gene in the transient-transfection assay may not be packaged appropriately into chromatin, we cannot rule out the possibility of the involvement of HDACs in AES function on endogenous genes within chromatin.

To further study the mechanism of action of AES, we performed additional protein-protein pull-down assays to assess possible interactions of AES with components of the basal transcriptional machinery. GST and GST-AES agarose beads were incubated with HeLa nuclear extract, and the bound proteins were analyzed by Western blot assays with polyclonal



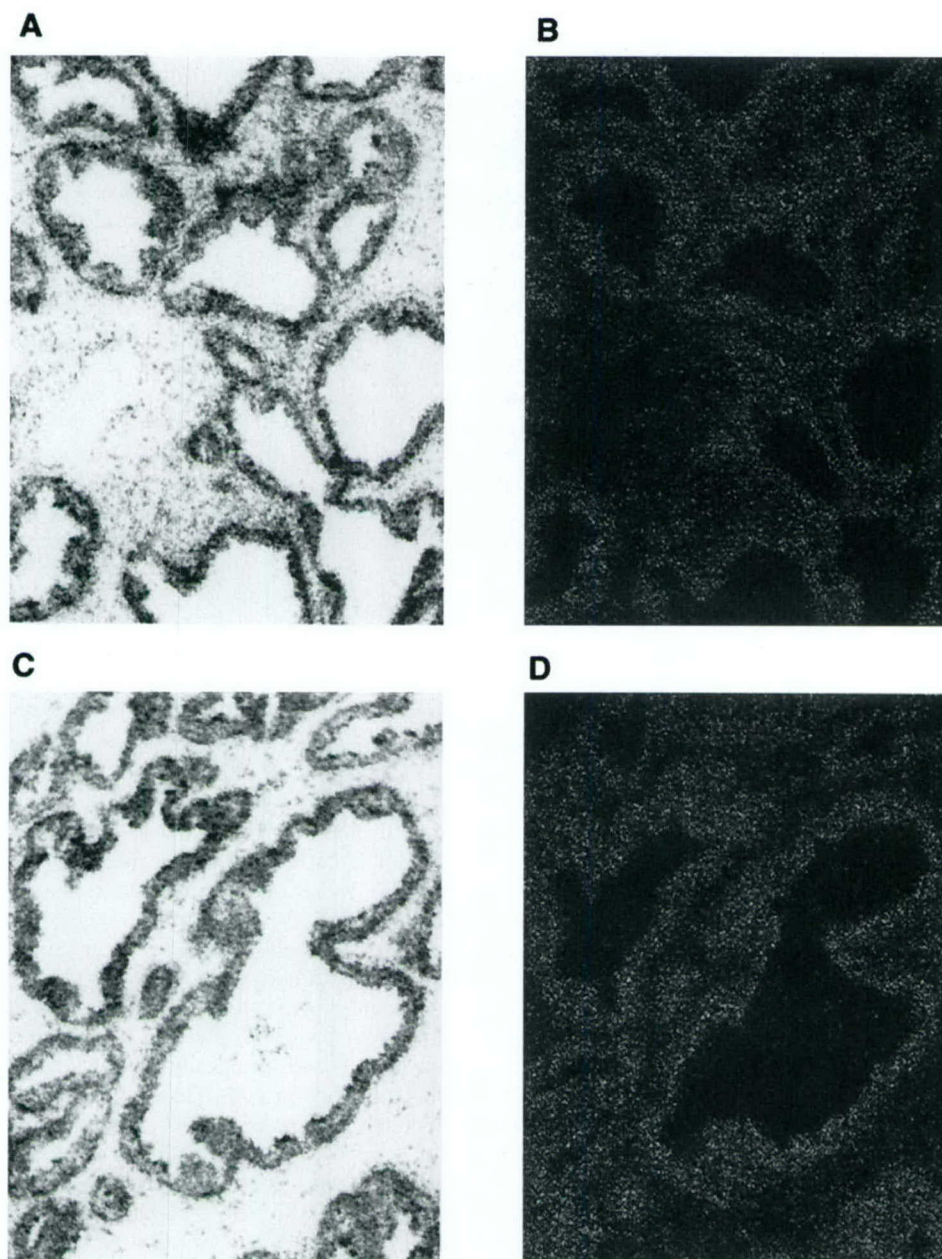


FIG. 7. AES expressed in the epithelial cells of the prostate. The slides containing sections of prostate tissues were hybridized with antisense AR (A and B) or AES (C and D) RNA probes. The emulsion-coated slides were exposed and evaluated under a Nikon microscope with a digital camera interfaced to a computer. The left and right panels show bright-field and dark-field images of the same area of the slides.

antibodies against subunits of RNA polymerase II and basal transcription factors. As shown in Fig. 6A, TFIIE (detected by antibodies against TFIIE $\alpha$ ) was specifically retained by GST-AES, relative to GST alone, whereas other basal transcription factors (TFIIA, TFIIB, TFIID, TFIIF, and TFIIH) and RNA polymerase II failed to be bound. These observations implicate TFIIE as a possible target for AES.

**AES is highly expressed, along with AR, in prostate epithelial cells.** To determine whether AES and AR are expressed in the same cells in humans, we investigated the expression levels of AES and AR in normal prostate tissues by *in situ* RNA hybridization. Consistent with previous observations (32), the

expression levels of AR were high in the epithelial cells of the prostate (Fig. 7A and B). Expression of AES was also evident in the epithelial cells (Fig. 7C and D). As negative controls, no hybridization signals above the background levels were detected with the sense RNA probes, thus indicating that the signals obtained with the antisense probe are specific (data not shown).

## DISCUSSION

Various cofactors that have been implicated in the function of AR, as well as a number of other nuclear receptors, include p300/CBP, p160 family proteins, the ARA group (ARA24,



ARA45, ARA54, ARA55, ARA70, and ARA160), ARIP3, SNURF, and BAG-1L (18, 19, 46). All of these enhance AR-mediated transcription in vivo, although there is not a clear mechanistic understanding of the function of these factors. The results described here demonstrate (i) that AES is a selective repressor of ligand-dependent AR-mediated transcription and (ii) that AES physically interacts with the N-terminal region of AR and represses AR-driven transcription by targeting the basal transcriptional machinery (possibly TFIIE). These observations thus reveal a new negative regulatory pathway for AR function, as well as new insights into the mechanism of action of mammalian Groucho/TLE proteins.

**AES represses AR-dependent transcription.** A number of proteins have been demonstrated to repress AR-dependent transcription in vivo. These include AP-1 (34, 47), NF- $\kappa$ B (39), TR4 (testicular orphan receptor 4) (24) and HBO1 (histone acetyl transferase binding to origin recognition complex 1) (49). AP-1, NF- $\kappa$ B, and TR4 appear to inhibit AR-dependent transcription by mutual transcriptional interference (unexpected interactions of distinct transcription factors). Although the molecular mechanisms that underlie this phenomenon have remained mostly elusive, this may involve competition for a coactivator commonly required by both activators (1).

HBO1 belongs to the MYST family, which is characterized by highly conserved C<sub>2</sub>HC zinc fingers and a putative histone acetyltransferase domain. HBO1 contains a putative repression domain, interacts with the DBD-LBD of AR, and inhibits AR-dependent transcription in vivo, although the exact mechanism of HBO1 action remains to be determined (49). Based on the results presented here, AES represses AR-driven transcription in a manner more like that of HBO1. Like HBO1, AES physically interacts with AR and specifically represses AR-dependent transcription in transient-transfection assays. Also as reported for HBO1, AES probably does not act broadly as a nuclear hormone receptor corepressor because it represses AR-dependent transcription but not TR- or ER-dependent transcription. These results are consistent with the fact that AES physically interacts with the N-terminal region of AR, which is not conserved in the N-terminal regions of TR and ER (37). Nonetheless, it remains important to determine whether AES might repress other (as yet untested) nuclear receptors and, related, whether other members of the Groucho/TLE family can repress the function of AR or other nuclear receptors.

Like AR, some of the DNA-binding partners for the Groucho/TLE proteins do not always act as transcriptional repressors, and, in fact, some are better characterized as activators (11). For the Groucho-interacting Dorsal and Runt domain proteins (2, 9, 20, 22), the context of the target gene promoter appears to be critical for determining whether activation or repression will occur. These observations suggest that the recruitment of Groucho/TLE proteins and/or their repressor activities might also be dependent on the nature of the target gene promoter. It is also possible that Groucho/TLE proteins might function as coactivators in certain situations. Thus, it will be important to determine whether AES repression of AR-driven transcription is dependent on the target gene promoter context.

**Mechanism of AES function.** At present, relatively little is known about the mechanisms by which Groucho/TLE family

proteins function as eukaryotic (co)repressors. Various repressors and activators recruit the Groucho/TLE proteins through specific interactions with various regions of Groucho proteins (11). In the well-defined reconstituted transcription system utilized here, we observed repression of AR-dependent transcription from DNA templates by recombinant AES. Consistent with the indication from this result that AES may function through interactions with the basal transcriptional machinery, a specific interaction of AES with the basal transcription factor TFIIE was observed. Similarly, previous studies have shown that the zinc finger protein Kruppel represses transcription through physical interactions with TFIIE (48). Hence, these studies suggest that TFIIE may serve as a more general target for various corepressors and repressors.

TUP1, a general transcriptional corepressor (21, 50), is a yeast analog of the Groucho/TLE proteins. Gromoller and Lehming (14) demonstrated that the essential holoenzyme component SRB7 is a physical and functional target of TUP1. In addition, genetic interactions between Cyc8-Tup1 and a variety of Pol II holoenzyme components (SRB8, SRB10, SRB11, Sin4, Rgr1, Rox3, and Hrs1) have been reported (23). However, we failed to detect direct interactions of AES with human SRB7 or the SRB7-containing TRAP/Mediator complex in protein-protein pull-down assays, indicating that human AES may not directly target human SRB7 or the TRAP/Mediator complex. This observation may reflect the fact that Tup1 and Groucho/TLE proteins show poor sequence conservation (at the amino acid level) in both repression domains and WD-40 repeats. Similar to AES and suggesting a chromatin-independent mechanism, the purified Tup1-containing complex directly represses transcription in a crude yeast extract in vitro (40).

Many corepressor complexes contain HDAC enzymes. The *Drosophila* HDAC Rpd3 has been identified as a Groucho-interacting protein (6), and, possibly related, Groucho proteins also interact with histone H3 (38). Yeast Tup1 similarly interacts directly and genetically with histones H3 and H4 (10, 38), and mutations in genes encoding the HDACs abolish Tup1-mediated repression (55). These findings have led to a repression model, possibly complementing the more direct mechanisms indicated above, involving Groucho/Tup1 recruitment by promoter-bound factors, HDAC recruitment by Groucho/Tup1, and subsequent function of HDAC to establish and/or maintain a transcriptionally silenced chromatin structure. Our results do not support this model for AES. First, we failed to detect interactions between AES and HDAC1- or HDAC3-containing complexes. Second, the deacetylase inhibitor TSA did not affect AES-mediated inhibition of AR-dependent transcription in transient-transfection assays. Third, we observed a direct inhibition of AR-dependent transcription by recombinant AES in a highly purified reconstituted transcription system on a naked DNA template.

In summary, our results point both to a novel function for AES in mediating repression of AR-dependent transcription and to a mechanism involving direct interactions both with AR and with the basal transcription machinery. AR is an important regulatory factor in the development, differentiation, and maintenance of male reproductive functions, as well as in the regulation of other sexually dimorphic processes ranging from the development of neural tissues to the modulation of im-



mune function (33). Thus, the mammalian Groucho-related protein AES, and possible other family members, may play a pivotal role in these biological processes by modulating the transcriptional activity of AR.

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#### REFERENCES

- Aarnisalo, P., J. J. Palvimo, and O. A. Janne. 1998. CREB-binding protein in androgen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA* **95**: 2122–2127.
- Aronson, B. D., A. L. Fisher, K. Blechman, M. Caudy, and J. P. Gergen. 1997. Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol. Cell. Biol.* **17**:5581–5587.
- Berger, S. L. 1999. Gene activation by histone and factor acetyltransferases. *Curr. Opin. Cell Biol.* **11**:336–341.
- Brinkmann, A. O., L. J. Blok, P. E. de Ruiter, P. Doesburg, K. Stekete, C. A. Berrevoets, and J. Trapman. 1999. Mechanisms of androgen receptor activation and function. *J. Steroid Biochem. Mol. Biol.* **69**:307–313.
- Chen, G., and A. J. Courey. 2000. Groucho/TLE family proteins and transcriptional repression. *Gene* **249**:1–16.
- Chen, G., J. Fernandez, S. Mische, and A. J. Courey. 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in *Drosophila* development. *Genes Dev.* **13**:2218–2230.
- Cleutjens, K. B., C. C. van Eekelen, H. A. van der Korput, A. O. Brinkmann, and J. Trapman. 1996. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J. Biol. Chem.* **271**:6379–6388.
- DeJong, J., R. Bernstein, and R. G. Roeder. 1995. Human general transcription factor TFIIA: characterization of a cDNA encoding the small subunit and requirement for basal and activated transcription. *Proc. Natl. Acad. Sci. USA* **92**:3313–3317.
- Duffy, J. B., and J. P. Gergen. 1994. Sex, segments, and the central nervous system: common genetic mechanisms of cell fate determination. *Adv. Genet.* **31**:1–28.
- Edmondson, D. G., M. M. Smith, and S. Y. Roth. 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. *Genes Dev.* **10**:1247–1259.
- Fisher, A. L., and M. Caudy. 1998. Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.* **12**:1931–1940.
- Fondell, J. D., H. Ge, and R. G. Roeder. 1996. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc. Natl. Acad. Sci. USA* **93**:8329–8333.
- Ge, H., E. Martinez, C. M. Chiang, and R. G. Roeder. 1996. Activator-dependent transcription by mammalian RNA polymerase II: in vitro reconstitution with general transcription factors and cofactors. *Methods Enzymol.* **274**:57–71.
- Gromoller, A., and N. Lehming. 2000. Srb7p is a physiological target of Tup1. *EMBO J.* **19**:6845–6852.
- Heinzel, T., R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W. M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**:43–48.
- Huang, E. Y., J. Zhang, E. A. Miska, M. G. Guenther, T. Kouzarides, and M. A. Lazar. 2000. Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes Dev.* **14**:45–54.
- Ito, M., C.-X. Yuan, S. Malik, W. Gu, J. D. Fondell, S. Yamamura, Z.-Y. Fu, X. Zhang, J. Qin, and R. G. Roeder. 1999. Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol. Cell* **3**:361–370.
- Janne, O. A., A. M. Moilanen, H. Poukka, N. Rouleau, U. Karvonen, N. Kotaja, M. Hakli, and J. J. Palvimo. 2000. Androgen-receptor-interacting nuclear proteins. *Biochem. Soc. Trans.* **28**:401–405.
- Jenster, G. 1999. The role of the androgen receptor in the development and progression of prostate cancer. *Semin. Oncol.* **26**:407–421.
- Jiang, J., H. Cai, Q. Zhou, and M. Levine. 1993. Conversion of a dorsal-dependent silencer into an enhancer: evidence for dorsal corepressors. *EMBO J.* **12**:3201–3209.
- Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson. 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68**:709–719.
- Kirov, N., L. Zhelnin, J. Shah, and C. Rushlow. 1993. Conversion of a silencer into an enhancer: evidence for a co-repressor in dorsal-mediated repression in *Drosophila*. *EMBO J.* **12**:3193–3199.
- Lee, M., S. Chatterjee, and K. Struhl. 2000. Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast. *Genetics* **155**:1535–1542.
- Lee, Y. F., C. R. Shyr, T. H. Thin, W. J. Lin, and C. Chang. 1999. Convergence of two repressors through heterodimer formation of androgen receptor and testicular orphan receptor-4: a unique signaling pathway in the steroid receptor superfamily. *Proc. Natl. Acad. Sci. USA* **96**:14724–14729.
- Li, J., J. Wang, Z. Nawaz, J. M. Liu, J. Qin, and J. Wong. 2000. Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. *EMBO J.* **19**:4342–4350.
- MacLean, H. E., G. L. Warne, and J. D. Zajac. 1997. Localization of functional domains in the androgen receptor. *J. Steroid Biochem. Mol. Biol.* **62**:233–242.
- Malik, S., and R. G. Roeder. 2000. Transcriptional regulation through mediator-like coactivators in yeast and metazoan cells. *Trends Biochem. Sci.* **25**:277–283.
- Mallo, M., P. M. Lieberma, and T. Gridley. 1995. Possible involvement of the mouse Grg protein in transcription. *Cell. Mol. Biol. Res.* **41**:435–440.
- Manova, K., K. Nocka, P. Besmer, and R. F. Bachvarova. 1990. Gonadal expression of *c-kit* encoded at the *W* locus of the mouse. *Development* **110**:1057–1069.
- Marivoet, S., P. van Dijk, G. Verhoeven, and W. Heyns. 1992. Interaction of the 90-kDa heat shock protein with native and in vitro translated androgen receptor and receptor fragments. *Mol. Cell. Endocrinol.* **88**:165–174.
- Martinez, E., H. Ge, Y. Tao, C. X. Yuan, V. Palhan, and R. G. Roeder. 1998. Novel cofactors and TFIIA mediate functional core promoter selectivity by the human TAFII150-containing TFIID complex. *Mol. Cell. Biol.* **18**:6571–6583.
- Miyamoto, K. K., S. A. McSherry, G. A. Dent, M. Sar, E. M. Wilson, F. S. French, Y. Sharief, and J. L. Mohler. 1993. Immunohistochemistry of the androgen receptor in human benign and malignant prostate tissue. *J. Urol.* **149**:1015–1019.
- Mooradian, A. D., J. E. Morley, and S. G. Korenman. 1987. Biological actions of androgens. *Endocr. Rev.* **8**:1–8.
- Murtha, P. E., W. Zhu, J. Zhang, S. Zhang, and C. Y. Young. 1997. Effects of Ca<sup>++</sup> mobilization on expression of androgen-regulated genes: interference with androgen receptor-mediated transactivation by AP-1 proteins. *Prostate* **33**:264–270.
- Nagy, L., H. Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber, and R. M. Evans. 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**:373–380.
- Oelgeschlager, T., Y. Tao, Y. K. Kang, and R. G. Roeder. 1998. Transcription activation via enhanced preinitiation complex assembly in a human cell-free system lacking TAFs. *Mol. Cell* **1**:925–931.
- Owen, G. I., and A. Zelent. 2000. Origins and evolutionary diversification of the nuclear receptor superfamily. *Cell Mol. Life Sci.* **57**:809–827.
- Palaparti, A., A. Baratz, and S. Stifani. 1997. The Groucho/transducin-like enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3. *J. Biol. Chem.* **272**: 26604–26610.
- Palvimo, J. J., P. Reinikainen, T. Ikonen, P. J. Kallio, A. Moilanen, and O. A. Janne. 1996. Mutual transcriptional interference between RelA and androgen receptor. *J. Biol. Chem.* **271**:24151–24156.
- Redd, M. J., M. B. Arnaud, and A. D. Johnson. 1997. A complex composed of tup1 and ssn6 represses transcription in vitro. *J. Biol. Chem.* **272**:11193–11197.
- Ren, B., K. J. Chee, T. H. Kim, and T. Maniatis. 1999. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev.* **13**:125–137.
- Roche, P. J., S. A. Hoare, and M. G. Parker. 1992. A consensus DNA-binding site for the androgen receptor. *Mol. Endocrinol.* **6**:2229–2235.
- Roeder, R. G. 1996. The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem. Sci.* **21**:327–335.
- Roeder, R. G. 1998. The role of general and gene-specific cofactors in the regulation of eukaryotic transcription. *Cold Spring Harbor Symp. Quant. Biol.* **63**:201–218.
- Roose, J., M. Molenaar, J. Peterson, J. Hurenkamp, H. Brantjes, P. Moerer, M. van de Wetering, O. Destree, and H. Clevers. 1998. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**:608–612.
- Sadar, M. D., M. Hussain, and N. Bruchovsky. 1999. Prostate cancer: molecular biology of early progression to androgen independence. *Endocr. Relat. Cancer* **6**:487–502.
- Sato, M., M. D. Sadar, N. Bruchovsky, F. Saatcioglu, P. S. Rennie, S. Sato, P. H. Lange, and M. E. Gleave. 1997. Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. *J. Biol. Chem.* **272**:17485–17494.



48. Sauer, F., J. D. Fondell, Y. Ohkuma, R. G. Roeder, and H. Jackle. 1995. Control of transcription by Kruppel through interactions with TFIIB and TFIIE beta. *Nature* **375**:162-164.
49. Sharma, M., M. Zarnegar, X. Li, B. Lim, and Z. Sun. 2000. Androgen receptor interacts with a novel MYST protein, HBO1. *J. Biol. Chem.* **275**: 35200-35208.
50. Smith, R. L., and A. D. Johnson. 2000. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem. Sci.* **25**:325-330.
51. Tetsuka, T., H. Uranishi, H. Imai, T. Ono, S. Sonta, N. Takahashi, K. Asamitsu, and T. Okamoto. 2000. Inhibition of nuclear factor-kappaB-mediated transcription by association with the amino-terminal enhancer of split, a Groucho-related protein lacking WD40 repeats. *J. Biol. Chem.* **275**:4383-4390.
52. Tsan, J. T., Z. Wang, Y. Jin, L.-Y. Hwang, R. O. Bash, and R. Baer. 1997. Mammalian cells as hosts for two-hybrid studies of protein-protein interaction, p. 217-232. *In* P. L. Barta and S. Fields (ed.), *The yeast two-hybrid system*. Oxford University Press, Oxford, United Kingdom.
53. Wang, B. Q., C. F. Kostrub, A. Finkelstein, and Z. F. Burton. 1993. Production of human RAP30 and RAP74 in bacterial cells. *Protein Expression Purif.* **4**:207-214.
54. Wang, Z., and R. G. Roeder. 1997. Three human RNA polymerase III-specific subunits form a subcomplex and participate in specific transcription initiation. *Genes Dev.* **11**:1315-1326.
55. Watson, A. D., D. G. Edmondson, J. R. Bone, Y. Mukai, Y. Yu, W. Du, D. J. Stillman, and S. Y. Roth. 2000. Ssn6-tup1 interacts with class I histone deacetylases required for repression. *Genes Dev.* **14**:2737-2744.
56. Xu, L., C. K. Glass, and M. G. Rosenfeld. 1999. Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.* **9**:140-147.
57. Yu, X., L. C. Wu, A. Bowcock, A. Aronheim, and R. Baer. 1998. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. *J. Biol. Chem.* **273**:25388-25392.
58. Yuan, C.-X., M. Ito, J. D. Fondell, Z.-Y. Fu, and R. G. Roeder. 1998. The TRAP220 component of a thyroid hormone receptor-associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. *Proc. Natl. Acad. Sci. USA* **95**:7939-7944.



# Heterogeneous Expression and Functions of Androgen Receptor Co-Factors in Primary Prostate Cancer

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**The androgen receptor (AR), a ligand-activated transcription factor of the steroid receptor superfamily, plays an important role in normal prostate growth and in prostate cancer. The recent identification of various AR co-factors prompted us to evaluate their possible roles in prostate tumorigenesis. To this end, we analyzed the expression of AR and eight of its co-factors by quantitative *in situ* RNA hybridization in 43 primary prostate cancers with different degrees of differentiation. Our results revealed nearly constant expression of AR and heterogeneous expression of AR co-factors, with increased expression of PIAS1 and Ran/ARA24, decreased expression of ELE1/ARA70, and no change in TMF1/ARA160, ARA54, SRC1, or TRAP220. Interestingly, whereas TMF1/ARA160, ELE1/ARA70, ARA54, RAN/ARA24, and PIAS1 were preferentially expressed in epithelial cells, another co-factor, ARA55, was preferentially expressed in stromal cells. Although the changes in levels of these co-activators did not correlate with Gleason score, their occurrence in high-grade prostatic intraepithelial neoplasia, suggests their involvement in initiation (or an early stage) of cancer. In addition, human prostate tumor cell proliferation and colony formation were markedly reduced by ELE1/ARA70. Together, these findings indicate that changes in levels of expression of AR co-factors may play important, yet different, roles in prostate tumorigenesis. (Am J Pathol 2002, 161:1467-1474)**

Androgens mediate development and maintenance of normal prostate tissue and also seem to be involved in prostate tumor growth and progression.<sup>1</sup> Androgens act through the androgen receptor (AR), which belongs to the large family of nuclear receptors.<sup>2</sup> These receptors are hormone-activated transcription factors and structurally conserved. Activation of AR by androgens is a multistep process that involves androgen binding to the re-

ceptor, an accompanying structural change in the receptor, loss of associated heat shock/chaperone proteins, translocation of the liganded receptor to the nucleus, and binding of the liganded receptor to target genes. There is increasing evidence that the transcriptional activity of AR and other nuclear receptors depends on their interaction with various co-factors (co-activators and co-repressors).<sup>3,4</sup> A variety of co-factors have been identified by their ability to bind various nuclear receptor domains and to alter the transcriptional activity of nuclear receptors after overexpression in cell lines.<sup>5</sup> The best-studied group includes p300/CBP, the p160 family (SRC-1, TIF-2/GRIP-1, ACTR/P-CIP),<sup>5</sup> and PCAF/GCN5 complexes (yeast SAGA, human STAGA).<sup>6,7</sup> All have histone acetyltransferase activities and are thought to act mainly through histone acetylation and consequent chromatin structural perturbations, although they can also act through functional acetylation of activators<sup>8</sup> and co-activators.<sup>9</sup> A second group includes the TRAP/DRIP/ARC/SMCC/mediator complex,<sup>10</sup> which shows subunit-specific interactions with both nuclear receptors (mainly through TRAP220) and other activators.<sup>10</sup> This complex in turn facilitates the function of RNA polymerase II and the general initiation factors on DNA templates at postchromatin-remodeling steps.<sup>10,11</sup> Of these various co-activators, p300/CBP<sup>12,13</sup> and p160s<sup>12,14-16</sup> have been shown to function with AR. Other co-factors implicated in the function of AR and, in most cases, other nuclear receptors, include the ARA group (ARA24, ARA54, ARA55, ARA70, and ARA160),<sup>17-20,35</sup> ARIP3,<sup>21</sup> SNURF,<sup>22</sup> and AES.<sup>23</sup>

Altered expression of nuclear hormone receptor co-factors has been implicated in the genesis and progression of breast cancers. Increased expression of TIF2, CBP, and steroid receptor RNA activator has been observed in breast tumor tissues.<sup>24-26</sup> Peroxisome proliferator-activated receptor-binding protein (PBP/TRAP220) and SRC3/AIB1 genes are frequently amplified and overexpressed in breast tumors.<sup>27,28</sup> In comparison, how-

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ever, little is known about the possibility of abnormal expression of co-factors in prostate cancer. Recently, Fujimoto and colleagues<sup>40</sup> found that the expression levels of ARA55 and SRC1 were higher in cancer specimens with a poor response to endocrine therapy than in those with a good response to endocrine therapy. To explore this question, we analyzed the levels of expression of both relatively AR-selective co-factors (TMF1/ARA160, ELE1/ARA70, ARA55, ARA54, Ran/ARA24, and PIAS1) and more general co-factors (SRC1, TRAP220) in human prostate cancer tissue by quantitative *in situ* hybridization. Among the tested co-factors, PIAS1 and RAN/ARA24 showed significantly higher expression levels in cancer tissue compared with benign tissue. In contrast, expression of ELE1/ARA70 was dramatically decreased in primary prostate tumor tissues. A subsequent analysis has demonstrated suppression of LNCaP cell growth by ELE1/ARA70. Collectively, these results imply that these co-factors likely play important but contrasting roles in prostate cancer differentiation and tumorigenesis.

## Materials and Methods

### Prostate Tissue Specimens and Pathological Evaluation

Prostate cancer and normal control tissues were derived from radical prostatectomy specimens of 43 prostate cancer patients treated at New York University Medical Center. The study protocol was approved by Institutional Review Board of New York University Medical Center. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of tissue (4  $\mu$ m) were cut and mounted on Superfrost Plus adhesion slides and used for histology, immunohistochemistry, and *in situ* hybridization. Prostate cancer foci were categorized as well differentiated (combined Gleason score 2 to 4;  $n = 9$ ), moderately differentiated (combined Gleason score 5 to 6;  $n = 17$ ), and poorly differentiated (combined Gleason score 7 and 8 to 10;  $n = 17$ ). The histological features and the Gleason score of each individual specimen were confirmed by two pathologists (JM and PL).

### Immunohistochemistry

The immunohistochemical staining was performed on an automated Ventana machine. Before staining, antigen retrieval was performed by heating the specimens in a microwave oven for 30 minutes in citrate buffer (pH 8.0) after dewaxing. A rabbit polyclonal anti-AR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was applied to the sections at a 1:100 dilution, and sections were then incubated overnight at 4°C. A streptavidin-biotin peroxidase detection system was used according to the manufacturer's instruction (DAKO, Carpinteria, CA), with 3,3'-diaminobenzidine as substrate.

**Table 1.** List of Oligonucleotide Primers Used for Polymerase Chain Reaction

Protein	Primer	Sequence (5'-3')	Size (bp)
AR	T3/2263	accaatgtcaactccaggatgct	499
	T7/2762	cttcaactgggtgtggaatagatg	
Ran/ARA24	T3/22	cagggtccagttcaactgtattggt	605
	T7/627	gagagcagttgtctgagcaacct	
ARA54	T3/863	ttgcccggtatgaccgc	474
ARA55	T7/1337	aaatgtttgtaagggttgcctagag	608
	T3/-20	ctggagactaccacctgcacatg	
ELE1/ARA70	T7/588	actgcagcagccccagc	491
	T3/1344	tgagcctgagaagcataaagattc	
TMF1/ARA160	T7/1835	acatctgtagaggagttcgatataac	581
	T3/477	ttcaggggaaacctgtggcag	
PIAS1	T7/1058	tatccctgtcctgacaattcatcat	638
	T3/7	gacagtgcggaactaaagcaaagt	
SRC1	T7/645	gaagtgtactcttctgtggacaactggt	499
	T3/3235	tatcagtcaccacatgaagc	
TRAP220	T7/3734	ggttattcagtcagtagctgctg	628
	T3/172	ttggtcagctgttggagacat	
	T7/800	ttgtacacagcagatgttcctca	

T3 primers contain the T3 RNA polymerase promoter sequence gcaattaaccctcactaaaggg at the 3' ends and T7 primers contain the T7 RNA polymerase promoter sequence cgtaatacagactcactataggg at the 5' ends. Numbers indicate position of the primers' 5' ends on the cDNA sequences (the A of the ATG translation start codon was arbitrarily given the number 1).

### In Situ Hybridization

The expression sequence tag (EST) cDNA clones of interest were obtained from Research Genetics, Inc (Carlsbad, CA). Oligonucleotides were designed to bear T7 promoter sequences on one end and T3 promoter sequences on the opposite end (Table 1) such that the sense and anti-sense probes were specified by the polymerase used. Fragments of corresponding genes (~500-bp DNA) were amplified using polymerase chain reaction. High-specific-activity <sup>33</sup>P-labeled RNA probes were synthesized by incubation of DNA with T7 or T3 RNA polymerase; 500  $\mu$ mol/L of GTP, ATP, and CTP; 3  $\mu$ mol/L of UTP; and 100  $\mu$ Ci  $\alpha$ -<sup>33</sup>P-UTP (6000 Ci/mmol) at 37°C for 45 minutes followed by DNase treatment for 15 minutes at 37°C. The probes were purified by chromatography on a Sephadex G-50 column. The yield and quality of the probes were assessed by trichloroacetic acid precipitation and scintillation counting, as well as by agarose gel electrophoresis and autoradiography.

After wax removal and rehydration, 4- $\mu$ m sections of formalin-fixed tissue were hybridized to the sense and anti-sense probes following the described procedures.<sup>30</sup> Then, slides were subjected to autoradiography by dipping in NTB-2 X-ray emulsion (Eastman-Kodak, Rochester, NY), exposed for 1 to 2 weeks, developed in D-19 developer (Eastman-Kodak), and fixed in G33C fixer. Lastly, the slides were counterstained with Gill's hematoxylin stain.

### Image and Statistical Analyses

The benign and malignant tissue components were compared on the same section to eliminate tissue-to-tissue and slide-to-slide variations of grain signals. We first ex-



amined the consistency of quantification of a given case by analyzing randomly selected areas of nonneoplastic and cancer foci in the cancer specimens for five cases. The results from block to block were comparable in all cases (data not shown). These results validated the *in situ* hybridization approach with formalin-fixed and paraffin-embedded tissues for analyzing the expression of AR and its co-factors. Slides were evaluated under a microscope (Nikon Eclipse E400) equipped with a digital camera (Princeton Scientific Instruments, Inc., Monmouth Junction, NJ) interfaced to a computer with IPLab software. The specimens were categorized into four groups according to degree of differentiation by Gleason score; Gleason score 4, Gleason score 5 to 6, Gleason score 7, and Gleason score 8 to 10. The grain (*in situ* signal) numbers from the areas of interest (cancer, prostatic intraepithelial neoplasia, and normal) were captured and counted using IPLab software, and divided by the number of cells to quantify as grain number/cell. An average of 30 to 50 cells were analyzed for each case. Differences in expression levels of the genes of interest among these four groups were subjected to nonparametric Kruskal-Wallis analysis of variance analyses. Results were further grouped according to changes in RNA levels (malignant versus benign tissue) of less than twofold and greater than twofold and analyzed by the chi-square test.

### Assays of Cell Growth Suppression

LNCaP cells were plated in six-well plates (35-mm wells) and grown in RPMI 1640 medium to ~70% confluency for transfection with plasmid DNA (pcDNA3.1 and pcDNA-ELE/ARA70) and 6  $\mu$ l of lipofectamine (Invitrogen Life Technologies, Carlsbad, CA). Two days later, cells were selected with G418 at 0.8  $\mu$ g/ml. After 4 weeks of selection with the medium change every 3 days, the cells were rinsed with phosphate-buffered saline (PBS), fixed with 2% formaldehyde in PBS for 15 minutes, stained with 0.5% crystal violet in PBS for 15 minutes, rinsed once or twice with distilled water, dried, and stored for subsequent quantification of colonies.

## Results

### Analysis of mRNA Expression by In Situ Hybridization

A large number of co-factors that regulate AR-driven transcription has been identified.<sup>4</sup> To determine possible relationships to prostate tumorigenesis and prostate cancer progression, we investigated the expression levels of various AR co-factors in normal and tumor prostate tissues by *in situ* hybridization. To validate the *in situ* hybridization results, we performed both *in situ* hybridization and immunohistochemical analysis for AR with the same set of slides from the formalin-fixed, paraffin-embedded tissue blocks. Strong and uniform immunostaining of AR was observed in the nuclei of both epithelial and stromal cells in the benign areas of each of the 43 specimens (Figure 1G). Consistently, AR mRNA expression levels were

high in epithelial cells and lower in stromal cells revealed by *in situ* hybridization (Figure 1, A and B). As a negative control, only background signals were detected with the sense AR probe (Figure 1C), indicating that the signals obtained with the anti-sense AR probe were specific.

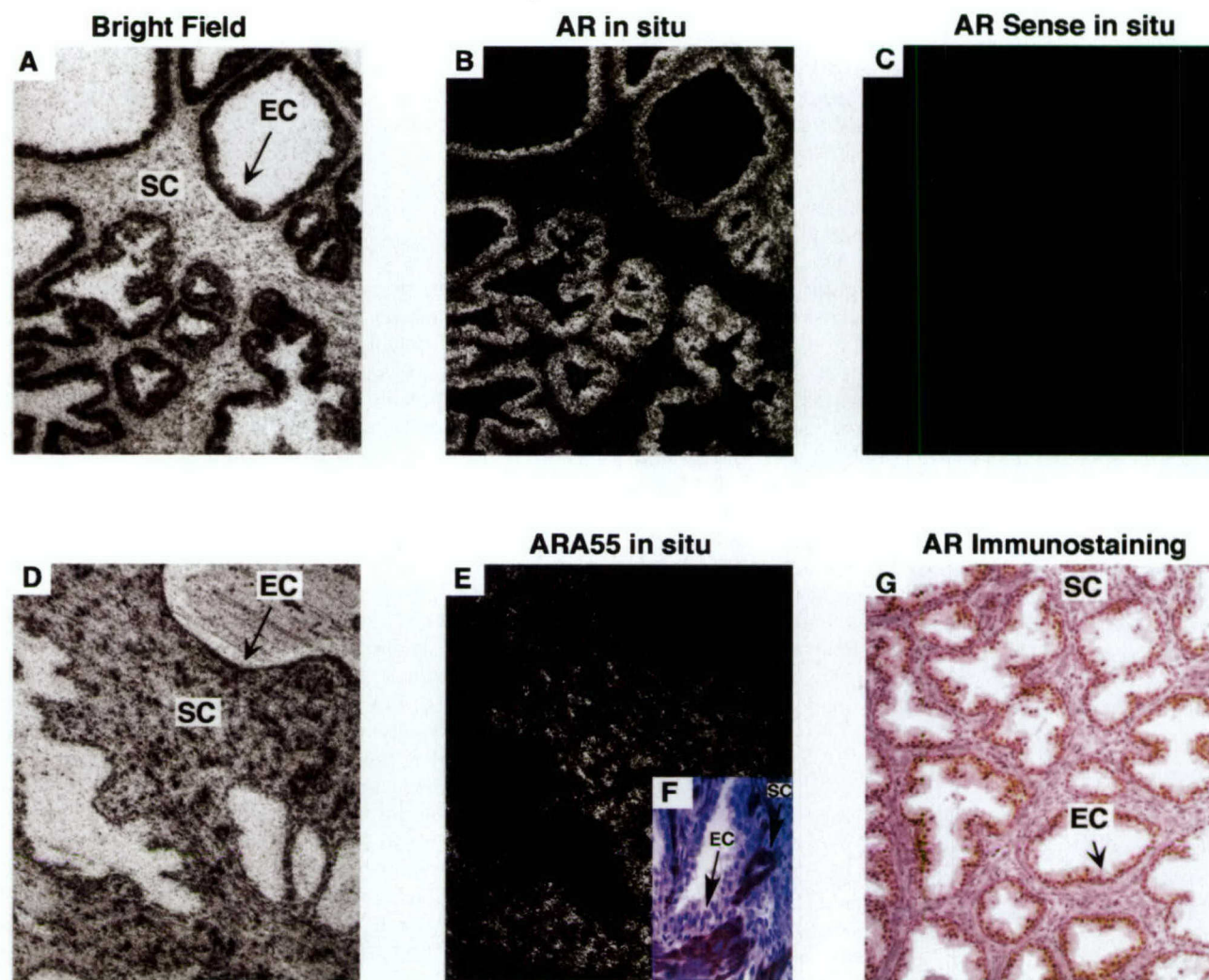
### Expression of AR and Co-Factor mRNAs: Epithelial Expression Versus Stromal Expression

In the 43 cases studied, the expression levels and overall expression patterns of AR did not differ significantly (less than twofold) between normal prostate tissue (from the same section of and adjacent to the prostate cancer region) and prostate cancer tissue. There was no apparent relationship between the amount of AR and the degree of tumor differentiation. These results are in accordance with previous reports that AR is highly expressed in a variety of normal and malignant human prostate tissues.<sup>31</sup> Expression levels of a panel of eight proteins described as modulators of AR function were then analyzed in the 43 prostate tumor samples. Two of these co-factors, SRC1 and TRAP220, interact with a broad spectrum of different nuclear receptors. The other six co-factors studied [the members of the ARA group (TMF1/ARA160, ELE1/ARA70, ARA55, ARA54 and Ran/ARA24) and PIAS1] are relatively specific for AR. The *in situ* hybridization results are summarized in Table 2. Expression of SRC1 and TRAP220 was detected in both stromal cells and epithelial cells, whereas expression of ARA54 was observed predominantly in epithelial cells. The expression levels of these mRNAs did not differ significantly between normal and tumor tissues. Although ARA55 mRNA was moderately expressed in stromal cells, it was undetectable in glandular epithelial cells (Figure 1, D and E), implying that ARA55 might regulate AR function in prostate stroma. Immunohistochemical staining with anti-ARA55 antibody on the same prostate tissues further confirmed that ARA55 was only expressed in stromal cells (Figure 1F). This is consistent with results of the previous study that ARA55 was detected only in cell lines derived from prostate stroma.<sup>32</sup> In most of the 43 specimens studied, the expression level of ARA55 was lower in the stroma in regions of cancerous foci however quantification was not possible because of the scanty nature of stroma in cancerous foci.

### Increased Expression of Ran/ARA24 and PIAS1 in Prostate Tumor Tissues

The 24-kd protein Ran/ARA24 belongs to the superfamily of GTP-binding proteins that use a structurally conserved G domain as a molecular switch for cycling between the GDP- and GTP-bound states.<sup>33</sup> Ran/ARA24 has been clearly implicated in the two-way traffic of macromolecules between the nucleus and the cytoplasm<sup>34</sup> and in microtubule assembly and spindle formation in cells in M phase. Recently, it has been shown that Ran/ARA24 physically interacts with the polyglutamine region of AR and enhances AR-dependent transcription.<sup>35</sup> Our *in situ*





**Figure 1.** Expression of AR and ARA55 in prostate tissues. **A** and **D**: Bright field (emulsion-coated) of AR and ARA55, respectively. **B** and **E**: Dark field of the same slide areas. **B** and **E** were hybridized with AR and ARA55 anti-sense probes, respectively, and **C** was hybridized with AR sense probes. **F** and **G**: Immunohistochemical analysis of ARA55 and AR in human prostate tissues. All slides were emulsion-coated, except **F** and **G**.

hybridization results for normal tissue showed only a low level of Ran/ARA24 mRNA expression that was present mainly in epithelial cells (Figure 2, A and B). Comparison of Ran/ARA24 expression in normal and tumor tissues

**Table 2.** Summary of *in Situ* Hybridization Data for AR and the Eight Examined Cofactors

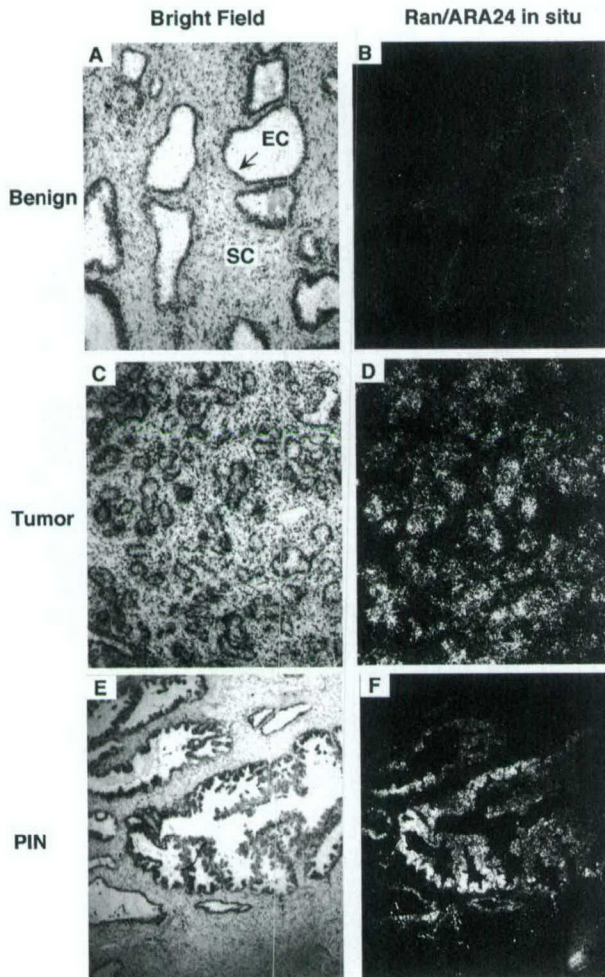
Factors	Stroma	Epithelium	
		Benign	Tumor
AR	++	+++	+++
SRC1	+	+	+
TMF1/ARA160	+/-	+	+
TRAP220	+/-	+	+
ARA55	++	-	-
ARA54	+/-	+	+
Ran/ARA24	+/-	+	+++
PIAS1	+/-	+	+++
ELE1/ARA70	-	+++	+

-, Indicates undetectable levels of expression; +/-, +, ++, and +++ indicate slightly above background, low, moderate, and high levels of expression, respectively.

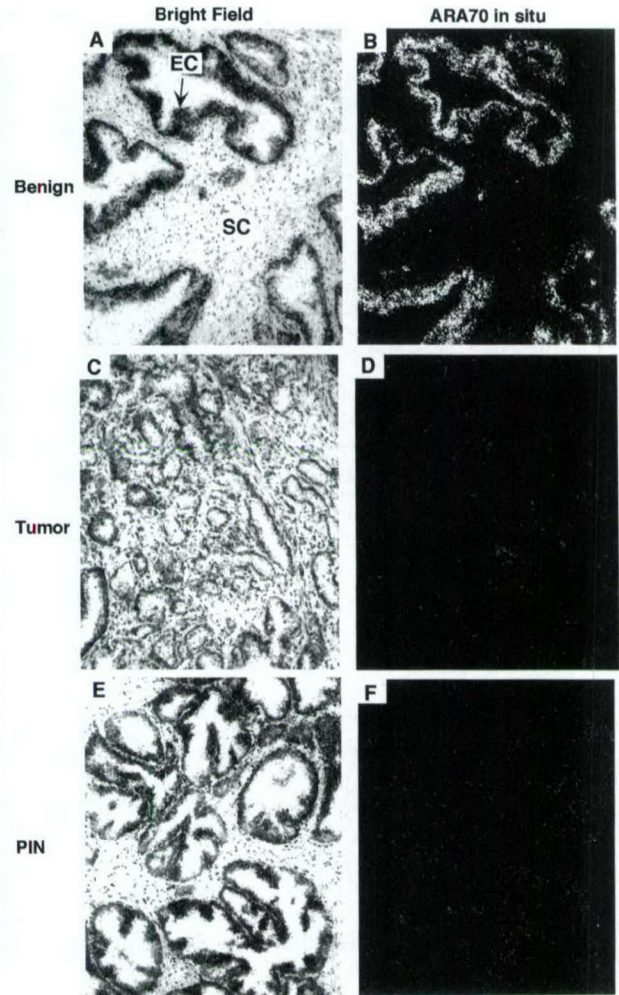
found overexpression (Figure 2, C and D *versus* A and B; Table 3) in 81% of the tumor specimens, with an average increase of  $4.6(\pm 1.1)$ -fold and no change in 19% of the specimens. More dramatic changes (more than fivefold) were observed in 35% of the tumor specimens (Table 3). However, these changes did not correlate with prostate tumor grade (Gleason score) by nonparametric Kruskal-Wallis analysis of variance analysis and by the chi-square test when cases were further grouped according to changes of less than twofold and greater than twofold.

PIAS1 has been identified as a factor that binds to Stat1 (signal transducer and activator of transcription 1) and inhibits STAT-mediated signaling by interfering with the DNA binding of Stat1.<sup>36</sup> PIAS1 has also been identified as a co-activator for AR-, estrogen receptor (ER)-, and progesterone receptor (PR)-dependent transcription.<sup>37,38</sup> In the current study, we observed higher PIAS1 expression levels in 33% of the tumor cases, with an average of 3.8-fold increase (Table 3). Although the percentage of cases showing an increase is significantly higher for Ran/ARA24 (81%) than for PIAS1 (33%), there





**Figure 2.** Increased expression of Ran/ARA24 in prostate cancer tissues. **Left** (A, C, E) and **right panels** (B, D, F) show bright and dark fields of the same areas of slides, respectively. A and B, C and D, and E and F show normal prostate, prostate tumor, and prostate intraepithelial neoplasia tissues, respectively. All slides are emulsion-coated.



**Figure 3.** Decreased expression of ELE1/ARA70 in prostate cancer tissues. **Left** and **right panels** show bright and dark fields of the same slide areas, respectively. A and B, C and D, and E and F show normal prostate, prostate tumor, and prostate intraepithelial neoplasia tissues, respectively. All slides are emulsion-coated slides.

is an 80% concordance between the increase for Ran/ARA24 and PIAS1. The PIAS1 expression patterns also did not correlate with prostate tumor grades by nonparametric Kruskal-Wallis analysis of variance and by the chi-square test.

### Lower Expression of ELE1/ARA70 in Prostate Cancer

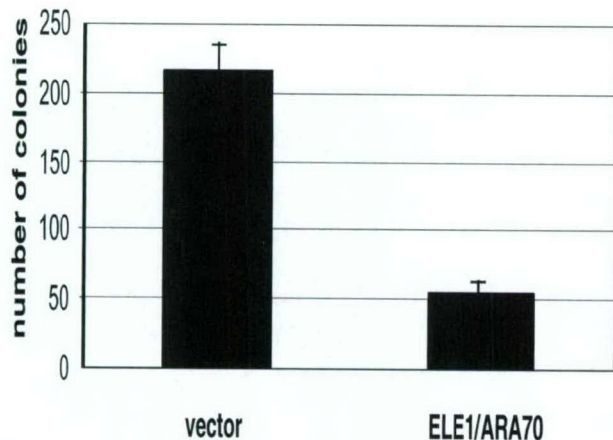
ELE1/ARA70 was identified first as a factor involved in the activation of the RET proto-oncogene in thyroid neoplasia<sup>39</sup> and later as a ligand-dependent transcriptional co-factor for AR.<sup>17</sup> Our *in situ* RNA hybridization assays showed that ELE1/ARA70, like AR, is expressed at high

levels (and predominantly in epithelial cells) in normal tissue (Figure 3, A and B). However, ELE1/ARA70 expression was dramatically lower in prostate tumor tissues (Figure 3, C and D versus A and B). Expression was decreased twofold to fivefold in 42% of the cases and 5- to 30-fold in 38% of cases (Table 3), with an average decrease of  $7.5(\pm 1.4)$ -fold. We further observed that, in cases with increased expression of RAN/ARA24 and reduced expression of ELE1/ARA70, 70% of the cases showed reciprocal changes, indicating opposite effects of these co-activators in cancer. No obvious correlation between ELE1/ARA70 expression and prostate tumor grade was observed by nonparametric Kruskal-Wallis analysis of variance analysis or by the chi-square test

**Table 3.** Quantification of PIAS1, Ran/ARA24, and ELE1/ARA70 Expression

TABLE 3. Quantitation of PIAS1, Ran/ARA24, and ELE1/ARA70								
	PIAS1			Ran/ARA24			ELE1/ARA70	
fold	<2	2-7.5	<2	2-5	5-20	<2	2-5	5-30
cases	24	12	8	20	15	9	18	16
%	67	33	19	46	35	20	42	38





**Figure 4.** Growth suppression of the prostate tumor cells by ELE1/ARA70. LNCaP prostate cancer cells were transfected with 4  $\mu$ g of pcDNA3.1 (vector) or pcDNA-ELE1/ARA70 and selected for plasmid-containing cells with G418 for 4 weeks. Surviving cells were then fixed and stained with crystal violet. Colonies were counted and the data are presented as histograms.

when cases were grouped according to changes less than twofold and greater than twofold.

#### *AR Co-Activator Expression in High-Grade Prostate Intraepithelial Neoplasia (HGPIN)*

HGPIN is thought to be a prostate cancer precursor lesion as a result of abundant evidence based on morphological, topographical, immunohistochemical, and molecular studies. HGPIN was identified in the majority of our cases (40 of 43 cases), either within or away from cancerous foci. A comparative analysis of AR co-factor expression patterns in HGPIN located adjacent to the prostate cancer region showed changes similar to those observed in prostate cancer. These changes included enhanced Ran/ARA24 expression (Figure 2, E and F versus A and B) and decreased ELE1/ARA70 expression (Figure 3, E and F versus A and B). We did not observe a significant difference in co-activator expression according to location of HGPIN. These results support the concept that HGPIN is a precursor of prostate cancer and further indicate that abnormal expression of Ran/ARA24 and ELE1/ARA70 may be involved in prostate tumor initiation.

#### *Suppression of Human Prostate Cancer Cell Proliferation and Colony Formation by ELE1/ARA70*

The decreased expression of ELE1/ARA70 in prostate cancer suggest that this co-factor might negatively regulate prostate cell growth and proliferation. We therefore tested the ability of ELE1/ARA70 gene to suppress the growth of prostate tumor cells, using the metastatic prostate cancer cell line LNCaP, which expresses reduced levels of ELE1/ARA70 compared with normal primary prostate epithelial cells.<sup>41</sup> Colony formation was suppressed by ELE1/ARA70 but not by the vector control (Figure 4). The colonies were small (containing a few

cells), even after 1 month of G418 selection (data not shown). These results indicate that ELE1/ARA70 suppresses tumor cell proliferation and colony formation and suggest that it may be a tissue differentiation factor or a potential tumor suppressor.

#### *Discussion*

Given the diverse functions of AR in different tissues, the large number of AR co-factors may provide means for cell- and promoter-specific regulation of AR activity.<sup>1,2</sup> Most co-factors are not receptor-specific but also regulate the activity of many nuclear receptors as well as unrelated transcription factors.<sup>3</sup> Furthermore, many co-factors are components of multiprotein complexes that have overlapping functions and nuclear receptor-binding sites.<sup>42</sup> The challenge is to identify co-factors involved in AR function in the prostate, particularly in prostate growth and prostate cancer progression. Our results demonstrate the heterogeneous expression and functions of AR co-factors in the prostate. Significantly, we observed increased expression of PIAS1 and Ran/ARA24 and decreased expression of ELE1/RAR70 both in prostate cancer tissues and in HGPIN, relative to normal prostate tissue. Furthermore, our *in vivo* studies using a malignant prostate cell line raise the possibility that ELE1/RAR70 might be a tumor suppressor.

#### *AR and Some Co-Factors Are Relatively Constant in Benign and Malignant Prostate Tissues*

Enhanced AR activity has been correlated with prostate cancer formation and progression.<sup>43</sup> It also has been proposed that either AR gene mutation or AR gene amplification may enhance AR activity, thus promoting tumorigenesis or leading to androgen-independent prostate cancer.<sup>1,44</sup> However, the relatively low incidence of AR mutation and amplification in primary prostate cancer suggests other causes. Consistent with this possibility, our *in situ* analyses have revealed that the levels and patterns of AR expression do not change significantly in primary prostate tumors of different grades. Down-regulation of SRC1, one of general nuclear receptor co-factors, is associated with tamoxifen resistance in breast neoplasms.<sup>45</sup> However, we did not detect a significant change in expression of SRC1 mRNA in prostate tumor tissue relative to normal tissue. TRAP220 was expressed in both epithelial and stromal cells, and the levels were not different in prostate cancer and benign prostate tissues. This might reflect rather broad functions of TRAP220 for various nuclear receptors and other activators.<sup>42</sup>

#### *Changed Expression of AR Co-Factors in Prostate Tumor*

Recent studies have shown that various co-activators can bind to AR and augment the AR transcription activity in a ligand-dependent manner.<sup>1,2</sup> Therefore, the activity of



co-factors might contribute to enhanced AR activity in primary prostate cancer. Our study shows that expression levels of Ran/ARA24 and PIAS1 are significantly higher in prostate tumor tissue compared with nonneoplastic prostate tissue. The higher levels of Ran/ARA24 and PIAS1 may contribute to overproliferation of prostate tumor cells. PIAS1 belongs to a family of PIAS proteins that, consistent with present results, are able to co-activate steroid receptor-dependent transcription.<sup>37,38</sup> In contrast to increased expression of RAN/ARA24 in 81% of prostate tumor cases, enhanced expression of PIAS1 was observed only in 33% of the cases. These differences may reflect the involvement of different pathways for Ran/ARA24 and PIAS1 as well as the relative efficiencies in contribution to cancer formation.

Interestingly, PIAS1 was first cloned as a protein that inhibited Stat1,<sup>38</sup> which has been suggested to have an anti-oncogenic effect.<sup>46</sup> This correlates with our findings that the level of PIAS1 is increased, possibly to revert the proapoptotic activity of Stat1, in prostate tissue. Further investigations will be needed to determine whether high levels of PIAS1 promote prostate cell proliferation through the Stat1 pathway, the AR pathway, or both pathways. Similar to PIAS1, Ran/ARA24 is also involved in nuclear translocation and chromatin organization. It is however unclear which pathways are affected by the enhanced expression of RAN/ARA24.

Previous studies demonstrated that ELE1/ARA70 can serve as a co-activator of AR, ER, PR, and peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ).<sup>17,29,47,48</sup> Here we report a down-regulation of ELE1/ARA70 expression in prostate cancer compared with levels expressed in nonneoplastic prostate tissues. Consistent with our observations, ELE1/ARA70 expression is reduced in prostate cancer cell lines relative to primary cells from benign prostate epithelium primary cells.<sup>41</sup> These observations suggest that ELE1/ARA70 may be involved in the development or progression of prostate cancer, particularly with respect to loss of androgen responsiveness. Overexpression of ELE1/ARA70 in a prostate cancer cell line suppresses cell proliferation and colony formation, suggesting that it might be a tumor suppressor or involved in the expression of genes required for prostate cell differentiation. AR in LNCaP cell harbors a mutation (codon 877, Thr to Ala) in the hormone-binding domain. This mutation confers an altered ligand-binding specificity.

The study of co-factor expression in prostate cancer should be of great importance for understanding AR function in prostate tumorigenesis and progression. A shift in the levels of various AR co-factors may influence the state of differentiation or proliferation of the prostate, possibly through the regulation of different AR responsive genes.

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### References

1. Jenster G: The role of androgen receptor in the development and progression of prostate cancer. *Semin Oncol* 1999, 26:407-421
2. Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketeer K, Berrevoets CA, Trapman J: Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* 1999, 69:307-313
3. Tsai SY, Tsai MJ, O'Malley BW: Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog Horm Res* 1997, 52:141-164
4. Janne OA, Moilanen A, Poukka H, Rouleau N, Karvonen U, Kotaja N, Hakli M, Palvimo JJ: Androgen-receptor-interacting nuclear proteins. *Biochem Soc Trans* 2000, 28:401-405
5. Xu L, Glass CK, Rosenfeld MG: Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* 1999, 9:140-147
6. Berger SL: Gene activation by histone and factor acetyltransferases. *Curr Opin Cell Biol* 1999, 11:336-341
7. Martinez E, Kundu TK, Fu J, Roeder RG: A human SPT3-TAF<sub>II</sub>31-GCN5-L acetylase complex distinct from TFIID. *J Biol Chem* 1998, 273:23781-23785
8. Gu W, Roeder RG: Activation of p53 sequence-specific DNA binding by acetylation of its C-terminal domain. *Cell* 1997, 90:595-606
9. Chen H, Lin RJ, Xie W, Wilpitz D, Evans RM: Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* 1999, 98:675-686
10. Malik S, Roeder RG: Transcriptional regulation through mediator-like coactivators in yeast and metazoan cells. *Trends Biochem Sci* 2000, 25:277-283
11. Fondell JD, Ge H, Roeder RG: Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc Natl Acad Sci USA* 1996, 93:8329-8333
12. Ikonen T, Palvimo JJ, Janne OA: Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. *J Biol Chem* 1997, 272:29821-29828
13. Aarnisalo P, Palvimo JJ, Janne OA: CREB-binding protein in androgen receptor-mediated signaling. *Proc Natl Acad Sci USA* 1998, 95:2122-2127
14. Berrevoets CA, Doesburg P, Steketeer K, Trapman J, Brinkmann AO: Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor 2). *Mol Endocrinol* 1998, 12:1172-1183
15. Ma H, Hong H, Huang SM, Irvine RA, Webb P, Kushner PJ, Coetzee GA, Stallcup MR: Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. *Mol Cell Biol* 1999, 19:6164-6173
16. Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B: The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. *Mol Cell Biol* 1999, 19:6085-6097
17. Yeh S, Chang C: Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc Natl Acad Sci USA* 1996, 93:5517-5521
18. Kang HY, Yeh S, Fujimoto N, Chang C: Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J Biol Chem* 1999, 274:8570-8576
19. Fujimoto N, Yeh S, Kang H-Y, Inui S, Chang H-C, Mizokami A, Chang C: Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. *J Biol Chem* 1999, 274:8316-8321
20. Hsiao PW, Chang C: Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. *J Biol Chem* 1999, 274:22373-22379
21. Moilanen AM, Karvonen U, Poukka H, Yan W, Toppari J, Janne OA, Palvimo JJ: A testis-specific androgen receptor coregulator that be-



- longs to a novel family of nuclear proteins. *J Biol Chem* 1999, 274: 3700–3704
22. Moilanen AM, Poukka H, Karvonen U, Häkli M, Jänne OA, Palvimo JJA: Identification of a novel RING finger protein as a coregulator in steroid receptor-mediated gene transcription. *Mol Cell Biol* 1998, 18:5128–5139
23. Yu X, Li P, Roeder RG, Wang Z: Inhibition of androgen receptor-mediated transcription by amino-terminal enhancer of split. *Mol Cell Biol* 2001, 21:4614–4625
24. Kurebayashi J, Otsuki T, Kunisue H, Tanaka K, Yamamoto S, Sonoo H: Expression levels of estrogen receptor- $\alpha$ , estrogen receptor- $\beta$ , coactivators, and corepressors in breast cancer. *Clin Cancer Res* 2000, 6:512–518
25. Murphy LC, Simon SL, Parkes A, Leygue E, Dotzlaw H, Snell L, Troup S, Adeyinka A, Watson PH: Altered expression of estrogen receptor coregulators during human breast tumorigenesis. *Cancer Res* 2000, 60:6266–6271
26. Leygue E, Dotzlaw H, Watson PH, Murphy LC: Expression of the steroid receptor RNA activator in human breast tumors. *Cancer Res* 1999, 59:4190–4193
27. Zhu Y, Qi C, Jain S, Le Beau MM, Espinosa R, Atkins GB, Lazar MA, Yeldandi AV, Rao MS, Reddy JK: Amplification and over-expression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. *Proc Natl Acad Sci USA* 1999, 96:10848–10853
28. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS: AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997, 277:965–968
29. Heinlein CA, Ting HJ, Yeh S, Chang C: Identification of ARA70 as a ligand-enhanced coactivator for the peroxisome proliferator-activated receptor gamma. *J Biol Chem* 1999, 274:16147–16152
30. Manova K, Nocka K, Besmer P, Bachvarova RF: Gonadal expression of c-kit encoded at the W locus of the mouse. *Development* 1990, 110:1057–1069
31. Miyamoto KK, McSherry SA, Dent GA, Sar M, Wilson EM, French FS, Sharief Y, Mohler JL: Immunohistochemistry of the androgen receptor in human benign and malignant prostate tissue. *J Urol* 1993, 149: 1015–1019
32. Nessler-Menardi C, Jtova I, Culig Z, Eder I, Putz T, Bartsch G, Klocker H: Expression of androgen receptor coregulatory proteins in prostate cancer and stroma-cell culture models. *Prostate* 2000, 45:124–131
33. Bourne HR, Sanders PD, McCormick F: The GTPase superfamily: a conserved structure and molecular mechanism. *Nature* 1991, 349: 117–127
34. Gorlich D, Kutay U: Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 1999, 15:607–660
35. Hsiao PW, Lin DL, Nakao R, Chang C: The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor poly-glutamine region-associated coactivator. *J Biol Chem* 1999, 274: 20229–20234
36. Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD, Shuai K: Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci USA* 1998, 95:10626–10631
37. Tan J, Hall SH, Hamil KG, Grossman G, Petrusz P, Liao J, Shuai K, French FS: Protein inhibitor of activated STAT-1 (signal transducer and activator of transcription-1) is a nuclear receptor coregulator expressed in human testis. *Mol Endocrinol* 2000, 14:14–26
38. Kotaja N, Aittomäki S, Silvennoinen O, Palvimo JJ, Janne OA: ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. *Mol Endocrinol* 2000, 14:1986–2000
39. Santoro M, Dathan NA, Berlingieri MT, Bongarzone I, Paulin C, Grieco M, Pierotti MA, Vecchio G, Fusco A: Molecular characterization of RET/PTC3: a novel rearranged version of the RET proto-oncogene in a human thyroid papillary carcinoma. *Oncogene* 1994, 9:509–516
40. Fujimoto N, Mizokami A, Harada S, Matsumoto T: Different expression of androgen receptor coactivators in human prostate. *Urology* 2001, 58:289–294
41. Tekur S, Lau KM, Long J, Burnstein K, Ho SM: Expression of RFG/ELE1 $\alpha$ /ARA70 in normal and malignant prostatic epithelial cell cultures and lines: regulation by methylation and sex steroids. *Mol Carcinog* 2001, 30:1–13
42. Ito M, Yuan CX, Malik S, Gu W, Fondell JD, Yamamura S, Fu Z-Y, Zhang X, Qin J, Roeder RG: Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol Cell* 1999, 3:361–370
43. Moul JW: Best screening tests for prostate cancer. *West J Med* 1998, 169:109–110
44. Hakimi JM, Rondinelli RH, Schoenberg MP, Barrack ER: Androgen-receptor gene structure and function in prostate cancer. *World J Urol* 1996, 14:329–337
45. Berns EM, van Staveren IL, Klijn JG, Foekens JA: Predictive value of SRC-1 for tamoxifen response of recurrent breast cancer. *Breast Cancer Res Treat* 1998, 48:87–92
46. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD: Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci USA* 1998, 95:7556–7561
47. Alen P, Claessens F, Schoenmakers E, Swinnen JV, Verhoeven G, Rombauts W, Peeters B: Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1 $\alpha$  with multiple steroid receptors and identification of an internally deleted ELE1 $\beta$  isoform. *Mol Endocrinol* 1999, 13:117–128
48. Gao T, Brantley K, Bolu E, McPhaul MJ: RFG (ARA70, ELE1) interacts with the human androgen receptor in a ligand-dependent fashion, but functions only weakly as a coactivator in cotransfection assays. *Mol Endocrinol* 1999, 13:1645–1656



## Identification of a Highly Conserved Domain in the Androgen Receptor That Suppresses the DNA-binding Domain-DNA Interactions\*

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**The androgen receptor (AR) is a ligand-regulated and sequence-specific transcription factor that activates or represses expression of target genes. Here, we show that the N terminus of AR contains an inhibitory domain located in an 81-amino acid segment lying upstream of the DNA-binding domain (DBD). The inhibitory domain interacted directly with DBD and repressed DBD binding to the androgen response element. Mutations of the conserved amino acid residues (K520E and R538E) within the inhibitory domain decreased its inhibiting ability *in vitro* and increased AR trans-activation *in vivo*. These data demonstrate the existence of a novel inhibitory domain in the N-terminal part of AR, which might play important roles in the regulation of AR trans-activation.**

The androgen receptor (AR)<sup>1</sup> mediates androgen functions in the differentiation and maturation of the male reproductive organs and in the development of male secondary sex characteristics (1). Mutations in the AR gene are associated with the androgen insensitivity syndrome (2, 3). Numerous somatic mutations in the AR gene have been reported among prostate cancer patients and as well as in prostate cancer cell lines and xenografts (3, 4). Most of these mutations have been detected in tumor tissues of late-stage prostate carcinoma, indicating that somatic mutation of the AR gene might be involved in the progression and aggressiveness of prostate cancer.

The AR is a member of the nuclear receptor superfamily (5). These receptors are characterized by distinct functional domains: an N-terminal part involved in ligand-independent transcription activation (AF1), a DNA-binding domain (DBD), and a C-terminal ligand-binding domain involved in ligand binding and ligand-dependent transcription activation (AF2) (6). As for other steroid receptors, ligand binding is generally believed to result in a conformational change in AR with con-

sequent dissociation of heat shock proteins/chaperones (7), dimerization, and binding to cognate androgen response elements (AREs) in target genes and (through its AF1 and AF2 domains) interactions with various coactivators that facilitate transcription by the general transcriptional machinery (8). The DBD encompasses two zinc finger-like modules and binds as dimers to two hexameric sequences orientated as direct or inverted repeats (9, 10). Although the DBD and the ligand-binding domain of steroid hormone receptors are highly conserved, there is much less homology among steroid hormone receptors in their N-terminal parts. The AR has a long N-terminal part with a strong autonomous AF1 and interacts directly with AF2 in the C-terminal part (11, 12). The N- and C-terminal interactions are important for androgen-induced gene regulation, and disruption of these interactions may be linked to androgen insensitivity syndrome (13, 14). The conserved FXXLF and WXXLF motifs within the N-terminal part seem to be involved in pairwise interactions between AF1 and AF2 (15). The N-terminal part contains stretches of glutamines (coded by CAG) and glycine (coded by GGN) (16). Expansion of the CAG repeats is associated with X-linked spinal and bulbar muscular atrophy (17). A shorter CAG repeat is associated with an increased trans-activation of AR (18, 19), but the biological role of GGN repeats is less clear.

In this study, we demonstrated that AR contains a highly conserved inhibitory domain within the N-terminal region. The inhibitory domain interacted directly with DBD and inhibited the DBD-DNA interactions. The mutations in the inhibitory domain resulted in decreased inhibitory ability and increased AR trans-activation activity, indicating that this domain might play important roles in the regulation of AR function.

### EXPERIMENTAL PROCEDURES

**Production and Purification of Recombinant Proteins**—The human full-length AR was expressed in Sf9 cells via the baculovirus expression vector pVL1393 (BD Biosciences), and the recombinant AR was purified as described previously (20). All of the AR and glucocorticoid receptor (GR) cDNA fragments were amplified by PCR with specific oligonucleotides, cut with *Nde*I and *Bam*HI, and subsequently cloned in the corresponding restriction sites of the vectors pET15d (Novagen), pGEX-2TL (Amersham Biosciences), and pCDNA3.1 (Invitrogen). The fragments were expressed as His<sub>6</sub>-tagged (via pET15d) or GST fusion (via pGEX-2TL) proteins in *Escherichia coli* BL21 and purified through nitrilotriacetic acid Ni<sup>2+</sup>-agarose or glutathione-Sepharose columns, respectively. Point mutations were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions and confirmed by DNA sequencing analysis. The mutated proteins were expressed and purified similarly.

**Gel Shift Assay**—Two pairs of oligomers (5'-AGCTTTTGCAGAACAGCAAGTGCTAGCTG-3' and 5'-AAATTCAGCTAGCACTTGCTGTTCTGCAA-3'; 5'-AGCTTTTGCAGAAATAGCAAATGCTAGCTG-3' and 5'-AAATTCAGCTAGCAATTTGCTATTCTGCAA-3') derived from the prostate-specific antigen gene (–152 to –174) were synthesized, annealed, and subcloned into *Hind*III and *Eco*RI sites of the vector pBlue-

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<sup>1</sup> The abbreviations used are: AR, androgen receptor; AF, transcription activation; DBD, DNA-binding domain; ARE, androgen response element; GR, glucocorticoid receptor; GST, glutathione S-transferase; Luc, luciferase; ID, inhibitory domain.



script II (Stratagene). The underlined bases were mutated from their corresponding bases in the wild-type prostate-specific antigen gene sequence. The wild-type and mutant ARE probes were made by cutting these constructs with *Xho*I and *Xba*I and purification of fragments from agarose gel. Probes were labeled with [ $\alpha$ - $^{32}$ P]dCTP by a fill-in reaction with the Klenow enzyme. In gel shift assays, 20- $\mu$ l reaction contains 20 mM HEPES, pH 7.9, 70 mM KCl, 1  $\mu$ g of poly(dI-dC), 1 mM dithiothreitol, 0.1% Nonidet P-40, 100  $\mu$ g/ml of bovine serum albumin, and various proteins. The reaction mixture was incubated for 20 min at room temperature, and the binding reaction was initiated by the addition of the labeled probes (20,000 cpm) and then incubated for an additional 30 min at room temperature. The reaction mixture was loaded directly onto a 4% (37.5:1, acrylamide:bisacrylamide) nondenaturing polyacrylamide gel with 0.25 $\times$  Tris borate EDTA and run at 150 V for 2 h at room temperature.

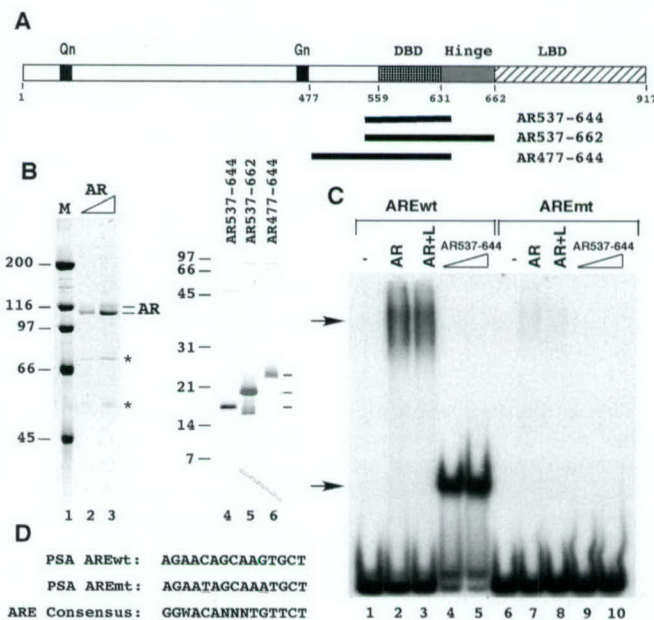
**Cell Culture and DNA Transfection**—PC3 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. Cells ( $5 \times 10^5$ ) were plated in each well of 24-well plates and transfected with 100 ng of 4xARE-E4-Luc reporter plasmid, 2.5 ng of control plasmid pRL-CMV, and various amounts of expression plasmids. Cells were grown in the presence of 10 nM R1881 for 48 h after transfection and harvested for dual-luciferase activity assay (Promega).

**Protein-Protein Pull-down Assay**—GST and GST-DBD(AR537–644) were expressed in bacteria and immobilized on glutathione-Sepharose beads. Beads (10  $\mu$ l) containing 100 ng of GST or GST-DBD proteins were incubated with 5  $\mu$ l of transcription and translation coupled rabbit reticulocyte lysates containing  $^{35}$ S-labeled AR477–538 in BC150, 0.1% Nonidet P-40 for 2 h at 4  $^{\circ}$ C. After being washed with the incubation buffer, beads were boiled with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography.

## RESULTS

**The Full-length AR Interacts with the Androgen Response Element More Weakly than the DNA-binding Domain**—The ligand-dependent interaction of AR with the ARE has been demonstrated *in vitro* with crude AR-containing cell extracts (21). However, the AR-DNA interactions have not been studied with the highly purified recombinant AR. To this end, the FLAG epitope-tagged human AR was expressed in Sf9 cells and immunopurified under high salt conditions (500 mM KCl) to strip off heat shock proteins associated with the unliganded AR. The recombinant AR preparation is near homogeneity (Fig. 1B, lanes 2 and 3) and contains two bands that migrated near the 110-kDa position. The *top band* might be the phosphorylated form of AR (22). Two minor polypeptides (70 and 55 kDa, indicated by *stars* on the right) were recognized by the anti-FLAG monoclonal antibody (data not shown), indicating that they are degraded products of the full-length AR. A DNA probe containing the ARE derived from the prostate-specific antigen promoter (–152 to –174) (Fig. 1D) (23) was used for a gel shift assay. The recombinant AR (0.9 pmol) shifted the probe (Fig. 1C, lane 2) while there was no ligand (androgen) dependence (lane 3 versus lane 2). The band of the AR:ARE complex (indicated by an arrow on the left) is quite broad. However, mutations of the nucleotides in the probe that are critical for AR-ARE interaction (24) (Fig. 1D) dramatically decreased the density of the AR-ARE band (Fig. 1C, lanes 7 and 8 versus lanes 2 and 3), indicating that the shifted band is specific. The DBD of AR (amino acid residues 537–644) (Fig. 1A) was expressed as a His<sub>6</sub>-tagged fusion protein and purified through an nitrilotriacetic acid Ni<sup>2+</sup>-agarose affinity column (Fig. 1B, lane 4). In the same assay, 0.3 pmol of AR537–644 almost completely shifted the probe (Fig. 1C, lane 4). The band of the AR537–644:ARE complex (indicated by an arrow on the left) is much sharper (Fig. 1C, lanes 4 and 5), and mutations in the probe (Fig. 1D) completely diminished the formation of the AR537–644:ARE complex (Fig. 1C, lanes 9 and 10). The results indicate that the binding affinity of DBD to the ARE is much stronger than that of the purified full-length AR to the same ARE.

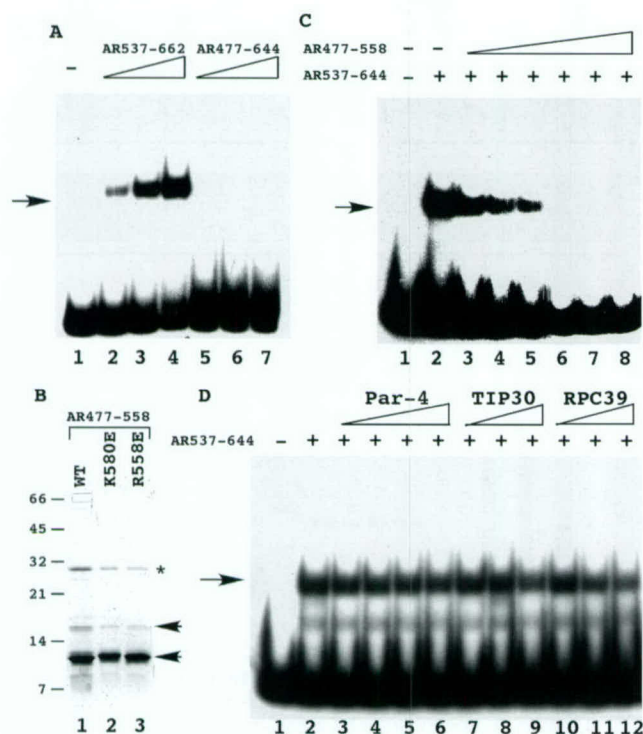
**A Domain within the AR N Terminus Inhibits DBD-ARE Interactions**—A C-terminal extension of the DBD of AR was



**FIG. 1. The recombinant AR interacts with the ARE weaker than the DBD.** A, diagram of domains and truncations of AR. B, SDS-PAGE analysis of the recombinant AR and DBD. Proteins of 100 ng (lane 2) and 200 ng (lane 3) of the purified recombinant AR expressed in Sf9 cells and recombinant His<sub>6</sub>-tagged truncations of AR expressed in bacteria (lanes 4–6) were subjected to SDS-PAGE with Coomassie Blue R250 staining. Lane 1 is standard protein markers (Bio-Rad). C, the gel shift assay was performed using a DNA probe containing the wild-type ARE (lanes 1–5) or the mutant ARE (lanes 6–10). 0.9 pmol of AR (lanes 2, 3, 7, and 8) and 0.3 pmol (lanes 4 and 9) or 0.6 pmol (lanes 5 and 10) of AR537–644 were used in the binding reactions. The synthetic androgen R1881 (100 nM) was included in the reactions in lanes 3 and 8, and lanes 1 and 6 are probes only. D, sequences of the wild-type (AREwt) and mutant (AREmt) ARE and the ARE consensus. The mutated bases in AREmt are underlined. PSA, prostate-specific antigen. M, standard molecular markers. Qn and Gn, stretches of glutamines and glycines, respectively. LBD, ligand-binding domain. AR+L, the gel shift assay performed in the presence of androgen R1881.

found to be required for specific and high affinity interactions of DBD with ARE (25). To investigate whether the sequences surrounding DBD would affect DBD-DNA interactions, AR537–662 and AR477–644 (Fig. 1A) were expressed in and purified from bacteria (Fig. 1B, lanes 5 and 6). AR537–662 strongly interacted with the probe, similar to AR537–644 (Fig. 2A, lanes 2–4). However, AR477–644 completely lost the ability to interact with the ARE probe even though much more protein (up to 1.6 pmol) was used in the binding reaction (lanes 5–7). The N-terminal extension of DBD (amino acid residues 477–558) was expressed and purified (Fig. 2B, lane 1). Its molecular mass as determined by SDS-PAGE (16 kDa) is much bigger than the calculated mass (10 kDa), and it was heavily degraded (Fig. 2B, lane 1). This region contains 20% charged amino acids and 16% proline residues, which may be responsible for this aberrant mobility of the protein. When AR477–558 was added to the binding reaction that contained the fixed amount (0.3 pmol) of AR537–644, the density of the DBD:ARE complex dramatically decreased (Fig. 2C, lanes 3–8). These results indicate that AR477–538 specifically inhibits the DBD-ARE interactions *in trans* as well as *in cis*. We noticed that different preparations of AR477–538 contained various amounts of the full-length protein and that amounts of the full-length protein (Fig. 2B, lane 1, indicated by the top arrow on the right) were correlated with the inhibition ability of AR477–538. As negative controls, the recombinant prostate apoptosis response-4 (26), 30-kDa Tat-interaction protein (27), and 39-kDa subunit of RNA polymerase C (28) expressed and



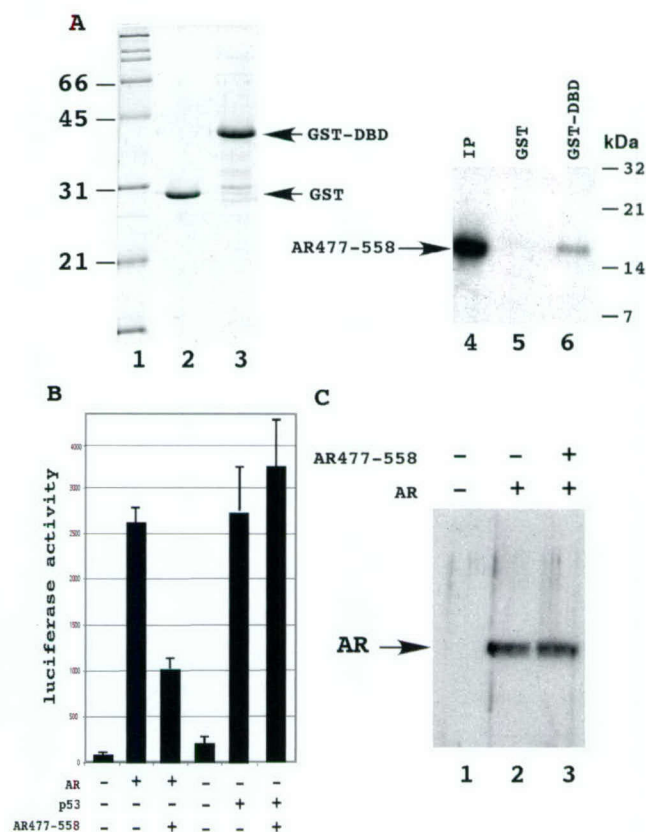


**FIG. 2. AR477-558 blocks DBD binding to ARE.** **A**, AR477-644 did not interact with the ARE. 0.15 (lane 2), 0.3 (lane 3), or 0.6 pmol (lane 4) of AR537-662 and 0.4 pmol (lane 5), 0.8 pmol (lane 6), or 1.6 pmol (lane 7) of AR477-644 were used in the binding reactions. Lane 1 is the probe-only control. **B**, SDS-PAGE of the purified recombinant wild-type (lane 1), K580E (lane 2), or R558E (lane 3) AR477-558. The full-length proteins (top arrow) and the main degraded products (bottom arrow) are indicated on the right. Nonspecific background bands are marked with a star on the right. **C**, AR477-558 inhibits AR537-643 binding to the ARE. The binding reactions contained 0.3 pmol of AR537-644 (lane 2) or 0.3 pmol of AR537-644 plus 0.0625 (lane 3), 0.125 (lane 4), 0.25 (lane 5), 0.5 (lane 6), 1 (lane 7), or 2 pmol (lane 8) of AR477-558. **D**, the purified recombinant PAR-4, TIP30, and RPC39 proteins do not affect the interaction of AR537-644 with ARE. The binding reactions contained 0.3 pmol of AR537-644 (lane 2) or 0.3 pmol of AR537-644 plus 0.225 (lane 3), 0.45 (lane 4), 0.9 (lane 5), or 1.8 pmol (lane 6) of prostate apoptosis response-4 (Par-4) (lane 8) or 1.25 (lanes 7 and 10), 2.5 (lanes 8 and 11), or 5 pmol (lanes 9 and 12) of 30-kDa Tat-interaction protein (TIP30) (lanes 7-9) or 39-kDa subunit of RNA polymerase C (RPC39) (lanes 10-12), respectively. WT, wild type.

purified similarly did not significantly affect the DBD binding to the ARE probe (Fig. 2D).

**The Inhibitory Domain Interacts with DBD and Inhibits AR Trans-activation**—The protein-protein pull-down assay was performed to investigate whether the inhibitory domain (ID) interacts directly with DBD. GST and GST-DBD(AR537-644) fusion protein were expressed in bacteria and immobilized on glutathione-Sepharose beads (Fig. 3A, lanes 2 and 3). The *in vitro* translated <sup>35</sup>S-labeled AR477-558 (lane 4) bound to GST-DBD (lane 6) and not to GST (lane 5). This result indicates that the inhibitory domain interacts directly with DBD.

We then investigated the effect of the ID on AR trans-activation by performing transient transfection assays. A luciferase reporter containing four tandem copies of the same ARE used for the gel shift assay upstream of the minimal adenovirus E4 promoter was cotransfected with expression vectors for AR, AR477-558, or both into prostate cancer PC3 cells in the presence of the synthetic androgen R1881. As shown in Fig. 3B, AR activated the reporter gene ~25-fold, and coexpressed AR477-558 showed a strong (62%) inhibition of this activity. Coexpression of AR477-558 did not influence reporter gene activity driven by p53, indicating that the inhibiting effect of AR477-558 was specific for AR. Western blot analysis revealed that the



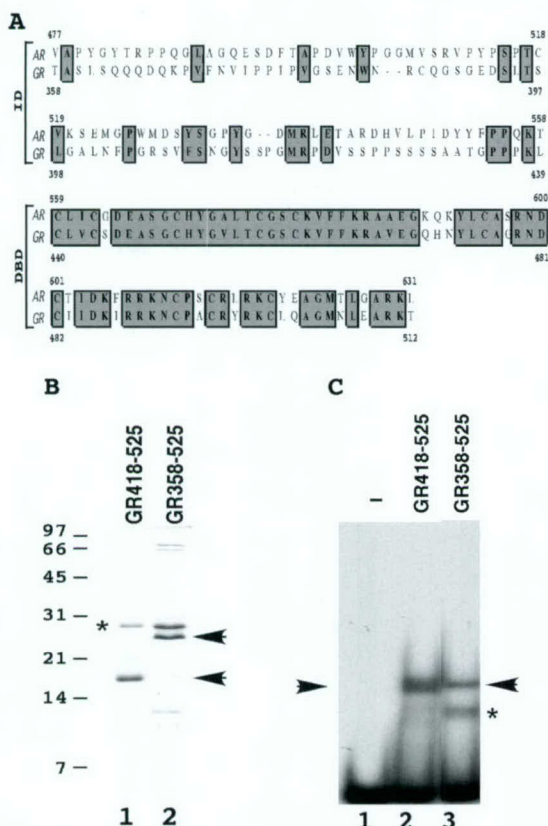
**FIG. 3. AR477-558 interacted directly with DBD *in vitro* and inhibited AR trans-activation *in vivo*.** **A**, AR477-558 interacted directly with DBD(AR537-644) *in vitro*. SDS-PAGE analysis of GST (lane 2) and GST-DBD (lane 3) expressed in bacteria and immobilized on glutathione-Sepharose 4B beads is shown. Bands corresponding to GST and GST-DBD fusion protein are indicated by arrows on the right. The immobilized GST-DBD pulled down <sup>35</sup>S-labeled AR477-558 (lane 6). Lane 4 is 10% <sup>35</sup>S-labeled AR477-558 input (IP) for the pull-down assay. **B**, AR477-558 inhibited AR trans-activation *in vivo*. PC3 cells were transfected with 100 ng of the reporters PGL3-ARE-E4 or pGL3-GAL4-E4, 2.5 ng of the internal control reporter pRL-CMV, 20 ng of pcDNA-AR or pcDNA-GAL4-p53-(1-53), and 18.5 ng of pcDNA-AR477-558 as indicated. Cells were treated with 10 nM R1881 after transfection and harvested 48 h later for the dual luciferase assay. **C**, AR477-558 did not affect AR protein levels in the transfected cells. Western blot analysis of cells transfected with pcDNA3.1 (lane 1), pcDNA-AR (lane 2), or pcDNA-AR plus pcDNA-AR477-558 (lane 3) with the anti-AR antibody is shown.

AR protein levels in the absence and presence of AR477-558 were comparable (Fig. 3C, lane 3 versus lane 2). On the basis of *in vitro* studies (Fig. 2), the ID inhibited AR trans-activation most likely by blocking the interaction of the AR with the ARE.

**The Inhibitory Domain Is Specific for AR**—The DNA-binding domains of AR, GR, progesterone receptor, and mineralocorticoid receptor are highly conserved (29). Not surprisingly, they bind to the same consensus DNA site (GGTACANNNTGTTCT) and can be considered a subfamily of the nuclear receptor superfamily. However, the inhibitory domain of AR is not conserved in the other receptors (Fig. 4A). GR418-525 and GR358-525 were expressed and purified (Fig. 4B, lanes 1 and 2). Gel shift assay demonstrated that GR358-525 and GR418-525 bound the ARE probe similarly (Fig. 4C, lane 3 versus lane 2). The lower band (Fig. 4C, lane 3, indicated by a star on the right) might contain a monomer of GR358-525. These results indicated that the ID in AR is not conserved in GR; thus, the inhibitory domain is specific for AR.

**Mutations in the ID Enhance AR Trans-activation**—Sequence alignment shows that the ID of AR is highly conserved through evolution (Fig. 5A). To further characterize the biolog-



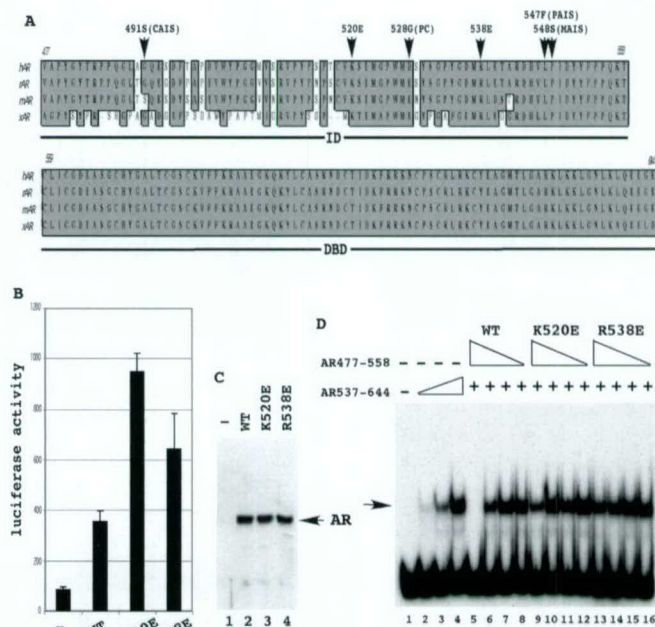


**FIG. 4. The DBD-containing fragments of GR bind to the ARE probe.** A, sequence alignment of DBD and ID of AR with the corresponding regions of rat GR. B, SDS-PAGE analysis of the recombinant GR418-525 and GR357-525. 500 ng of His<sub>6</sub>-tagged GR418-525 (lanes 1) and GR 357-525 (lane 2) expressed in bacteria were subjected to SDS-PAGE with Coomassie Blue R250 staining. The bands corresponding to the full-length protein fragments are indicated by arrows on the right, and a nonspecific background band is marked by a star on the left. The standard protein markers (Bio-Rad) are indicated on the left. C, GR417-525 and GR357-525 bind to the ARE. 0.3 pmol of GR417-525 (lane 2) or GR357-525 (lane 3) was used in the binding reactions. Lane 1 is the probe-only control.

ical effects of this region, we mutated two conserved residues (Lys-520 and Arg-538) in the ID and cDNAs encoding the mutated AR (K520E and R538E) were transiently transfected in PC3 cells with the luciferase reporter plasmid. The mutated AR had elevated trans-activation activity compared with the wild-type AR (Fig. 5B), although the mutated and wild-type AR were expressed at the same level in the transfected cells (Fig. 5C, lanes 2-4). The ID (AR477-558) from the mutated AR (K520E and R538E) were expressed and purified (Fig. 2B, lanes 2 and 3). The gel shift assay revealed that mutations of K520E and R538E decreased the inhibitory ability of ID (Fig. 5D, lanes 9-12 and 13-16 versus lanes 5-8). Ten nanograms of the wild-type AR477-558 almost completely blocked AR537-644 binding to the ARE probe (Fig. 5D, lane 5). However, the same amount of the mutated (K520E and R538E) AR477-558 inhibited the DBD-ARE interaction only 65 and 35%, respectively (Fig. 5D, lanes 9 and 13). Thus, the enhancement of AR trans-activation by mutations of K520E and R538E correlates with a decrease in the inhibitory effect of ID on DBD-ARE interactions.

#### DISCUSSION

The N-terminal parts of nuclear receptors are the most divergent among members of this superfamily of proteins, suggesting that each receptor will take on a unique N-terminal conformation to determine its specificity. This paper describes



**FIG. 5. Mutations in ID enhanced AR trans-activation and decreased ID inhibitory activity.** A, sequence alignment of ID and DBD of human (hAR), rabbit (rAR), mouse (mAR), and Xenopus (xAR) AR. Point mutations found in prostate cancer (PC) and in complete (CAIS), mild (MIAS), or partial (PAIS) androgen insensitivity syndrome patients are indicated by arrows on the top. B, mutations (K520E and R538E) enhanced AR trans-activation *in vivo*. PC3 cells were transfected with 100 ng of the reporter pGL3-ARE-E4, 2.5 ng of the internal control reporter pRL-CMV, or 10 ng of pcDNA-wild-type AR or pcDNA-mutant (K520E or R538E) AR as indicated. Cells were treated with 10 nM R1881 after transfection and harvested 48 h later for the dual luciferase assay. Each value represents the mean  $\pm$  S.D. of a representative experiment performed in triplicate. C, Western blot analysis of cells transfected with pcDNA3.1 (lane 1) or with wild-type (lane 2), K520E (lane 3), and R538E (lane 4) mutant AR. D, mutations of K520E and R538E decreased ID inhibitory ability on DBD-ARE interactions. The binding reactions contained 0.075 (lane 2), 0.15 (lane 3), or 0.3 pmol (lane 4) of DBD alone or 0.3 pmol of DBD plus 0.625 (lanes 5, 9, and 13), 0.125 (lanes 6, 10, and 14), 0.025 (lanes 7, 11, and 15), or 0.005 pmol (lanes 8, 12, and 16) of wild-type (lanes 5-8), K520E mutant (lanes 9-12) or R538E mutant AR477-558 (lanes 13-16). WT, wild type.

a highly conserved novel inhibitory domain designated ID, which lies in N-terminal 81-amino acid residues upstream of the DBD of AR. ID interacts directly with DBD and strongly inhibits the DBD-ARE interactions *in vitro* and AR trans-activation *in vivo*.

Much of the work devoted to understanding regulation of transcription by the AR has focused on the N-terminal AF1 and the C-terminal AF2 (30). However, transcriptional inhibition may be equally important as a way of preventing activation. Studies that deal with inhibition of AR-dependent transcription have focused on silencing mechanisms through recruitment of corepressors to the target promoters and through receptor occupancy at one DNA site interfering with transcription by an activator at an adjoining site (5, 31). We have now demonstrated that negative function element exists in the AR molecule itself and markedly suppresses the DNA binding activity of DBD. The ID function is similar to that of the N-terminal region of TAF250 (the 250-kDa TATA box-binding protein-associated factor 1), which forms a DNA-like structure, interacts with the DNA-binding surface, and inhibits the DNA binding activity of TATA box-binding protein (32). In contrast, the direct interactions between the ID and DBD suggest that perhaps ID acts through intramolecular contacts. In this respect, ID is similar to p53, which exists in a latent



DNA-binding form as a result of the C-terminal tail-DNA-binding domain interactions (33, 34). Phosphorylation of lysine residues in the C-terminal region leads to the disruption of interactions between the C-terminal domain and the core DBD, thus allowing the DBD of p53 to adopt an active conformation. It is important to know whether modifications in the ID of AR or interactions of this domain with the other proteins might regulate the DNA binding activity of AR. A study on the rat AR indicated that the unknown protein could enhance the DNA binding activity of the protein fragment containing the DBD in a gel shift assay (35). Another study has demonstrated that mutations on <sup>668</sup>QPIF<sup>671</sup> at the boundary of the hinge and ligand-binding domain of AR, resulting in receptors that exhibit 2–4-fold increased activity compared with the wild-type AR in response to dihydrotestosterone, and these mutations have been detected in prostate cancer patients (36). However, the molecular mechanism for this phenomenon is unclear.

Several mutations found in men with prostate cancer (37) and in men with the androgen insensitivity syndrome (38, 39) localize in ID (Fig. 5A). These mutations might change the function of ID, therefore affecting AR trans-activation. D528G mutation was detected in a patient with prostate cancer (37), and we found that AR with D528G mutation was more active (>3-fold) than the wild-type AR in transient transfection assays (data not shown). Currently, we are investigating whether the enhanced AR trans-activation is because of the decreased ID function. Thus, ID may play an important regulatory role in AR function, and dysfunction of ID may contribute to prostate cancer or androgen insensitivity syndrome in some men.

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#### REFERENCES

- Gelmann, E. P. (2002) *J. Clin. Oncol.* **20**, 3001–3015
- Brinkmann, A. O., Jenster, G., Ris-Stalpers, C., van der Korput, J. A., Bruggenwirth, H. T., Boehmer, A. L., and Trapman, J. (1995) *J. Steroid Biochem. Mol. Biol.* **53**, 443–448
- Culig, Z., Hobisch, A., Hittmair, A., Cronauer, M. V., Radmayr, C., Bartsch, G., and Klocker, H. (1997) *Drugs Aging* **10**, 50–58
- Culig, Z., Klocker, H., Bartsch, G., and Hobisch, A. (2001) *Am. J. Pharmacogenomics* **1**, 241–249
- Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) *Curr. Opin. Genet. Dev.* **9**, 140–147
- Weatherman, R. V., Fletterick, R. J., and Scanlan, T. S. (1999) *Annu. Rev. Biochem.* **68**, 559–581
- Heinlein, C. A., and Chang, C. (2001) *Endocrine* **14**, 143–149
- Heinlein, C. A., and Chang, C. (2002) *Endocr. Rev.* **23**, 175–200
- Melvin, V. S., Roemer, S. C., Churchill, M. E., and Edwards, D. P. (2002) *J. Biol. Chem.* **277**, 25115–25124
- Schoenmakers, E., Verrijdt, G., Peeters, B., Verhoeven, G., Rombauts, W., and Claessens, F. (2000) *J. Biol. Chem.* **275**, 12290–12297
- Berrevoets, C. A., Doesburg, P., Steketee, K., Trapman, J., and Brinkmann, A. O. (1998) *Mol. Endocrinol.* **12**, 1172–1183
- Jenster, G., van der Korput, H. A., Trapman, J., and Brinkmann, A. O. (1995) *J. Biol. Chem.* **270**, 7341–7346
- He, B., and Wilson, E. M. (2002) *Mol. Genet. Metab.* **75**, 293–298
- Thompson, J., Saatcioglu, F., Janne, O. A., and Palvimo, J. J. (2001) *Mol. Endocrinol.* **15**, 923–935
- He, B., Minges, J. T., Lee, L. W., and Wilson, E. M. (2002) *J. Biol. Chem.* **277**, 10226–10235
- Csaszar, A., and Abel, T. (2001) *Eur. J. Pharmacol.* **414**, 9–22
- McEwan, I. J. (2001) *Biochem. Soc. Trans.* **29**, 222–227
- Kantoff, P., Giovannucci, E., and Brown, M. (1998) *Biochim. Biophys. Acta* **1378**, C1–C5
- Nelson, K. A., and Witte, J. S. (2002) *Am. J. Epidemiol.* **155**, 883–890
- Yu, X., Li, P., Roeder, R. G., and Wang, Z. (2001) *Mol. Cell. Biol.* **21**, 4614–4625
- Wong, C. I., Zhou, Z. X., Sar, M., and Wilson, E. M. (1993) *J. Biol. Chem.* **268**, 19004–19012
- Gioeli, D., Ficarro, S. B., Kwiek, J. J., Aaronson, D., Hancock, M., Catling, D. A., White, F. M., Christian, R. E., Settlege, R. E., Shabanowitz, J., Hunt, D. F., and Weber, M. J. (2002) *J. Biol. Chem.* **277**, 29304–29314
- Cleutjens, K. B., van Eekelen, C. C., van der Korput, H. A., Brinkmann, A. O., and Trapman, J. (1996) *J. Biol. Chem.* **271**, 6379–6388
- Roche, P. J., Hoare, S. A., and Parker, M. G. (1992) *Mol. Endocrinol.* **6**, 2229–2235
- Schoenmakers, E., Alen, P., Verrijdt, G., Peeters, B., Verhoeven, G., Rombauts, W., and Claessens, F. (1999) *Biochem. J.* **341**, 515–521
- Sells, S. F., Han, S. S., Muthukumar, S., Maddiwar, N., Johnstone, R., Boghaert, E., Gillis, D., Liu, G., Nair, P., Monnig, S., Collini, P., Mattson, M. P., Sukhatme, V. P., Zimmer, S. G., Wood, D. P., Jr., McRoberts, J. W., Shi, Y., and Rangnekar, V. M. (1997) *Mol. Cell. Biol.* **17**, 3823–3832
- Xiao, H., Tao, Y., Greenblatt, J., and Roeder, R. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2146–2151
- Wang, Z., and Roeder, R. G. (1997) *Genes Dev.* **11**, 1315–1326
- Forman, B. M., and Samuels, H. H. (1990) *Mol. Endocrinol.* **4**, 1293–1301
- Bevan, C. L., Hoare, S., Claessens, F., Heery, D. M., and Parker, M. G. (1999) *Mol. Cell. Biol.* **19**, 8383–8392
- Lobaccaro, J. M., Poujol, N., Terouanne, B., Georget, V., Fabre, S., Lumbroso, S., and Sultan, C. (1999) *Endocrinology* **140**, 350–357
- Liu, D., Ishima, R., Tong, K. I., Bagby, S., Kokubo, T., Muhandiram, D. R., Kay, L. E., Nakatani, Y., and Ikura, M. (1998) *Cell* **94**, 573–583
- Jayaraman, J., and Prives, C. (1995) *Cell* **81**, 1021–1029
- Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83**, 237–245
- Kupfer, S. R., Marschke, K. B., Wilson, E. M., and French, F. S. (1993) *J. Biol. Chem.* **268**, 17519–17527
- Buchanan, G., Yang, M., Harris, J. M., Nahm, H. S., Han, G., Moore, N., Bentel, J. M., Matusik, R. J., Horsfall, D. J., Marshall, V. R., Greenberg, N. M., and Tilley, W. D. (2001) *Mol. Endocrinol.* **15**, 46–56
- Tilley, W. D., Buchanan, G., Hickey, T. E., and Bentel, J. M. (1996) *Clin. Cancer Res.* **2**, 277–285
- Ahmed, S. F., Cheng, A., Dovey, L., Hawkins, J. R., Martin, H., Rowland, J., Shimura, N., Tait, A. D., and Hughes, I. A. (2000) *J. Clin. Endocrinol. Metab.* **85**, 658–665
- Sutherland, R. W., Wiener, J. S., Hicks, J. P., Marcelli, M., Gonzales, E. T., Jr., Roth, D. R., and Lamb, D. J. (1996) *J. Urol.* **156**, 828–831



## Purification and Identification of a Novel Complex Which Is Involved in Androgen Receptor-Dependent Transcription

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**The androgen receptor (AR) binds to and activates transcription of target genes in response to androgens. In an attempt to isolate cofactors capable of influencing AR transcriptional activity, we used an immunoprecipitation method and identified a 44-kDa protein, designated p44, as a new AR-interacting protein. p44 interacts with AR in the nucleus and with an androgen-regulated homeobox protein (NKX3.1) in the cytoplasm of LNCaP cells. Transient-transfection assays revealed that p44 enhances AR-, glucocorticoid receptor-, and progesterone receptor-dependent transcription but not estrogen receptor- or thyroid hormone receptor-dependent transcription. p44 was recruited onto the promoter of the prostate-specific antigen gene in the presence of the androgen in LNCaP cells. p44 exists as a multiprotein complex in the nuclei of HeLa cells. This complex, but not p44 alone, enhances AR-driven transcription in vitro in a cell-free transcriptional system and contains the protein arginine methyltransferase 5, which acts synergistically with p44 to enhance AR-driven gene expression in a methyltransferase-independent manner. Our data suggest a novel mechanism by which the protein arginine methyltransferase is involved in the control of AR-driven transcription. p44 expression is dramatically enhanced in prostate cancer tissue compared with adjacent benign prostate tissue.**

The androgen receptor (AR) mediates androgen function in the development and maintenance of normal prostate tissue (4). The growth and progression of prostate cancer are also dependent on AR. AR is a member of the nuclear receptor superfamily and, like other members of this family, contains a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) with an associated activation function (AF-2) activation domain, and an N-terminal domain (NTD) containing the AF-1 activation domain (9, 20). On ligand binding, AR dissociates from heat shock proteins and chaperones, dimerizes, binds to cognate androgen response elements (AREs) in target genes, and, through its AF-1 and AF-2 domains, interacts with various coactivators that facilitate transcription by the general transcriptional machinery (9, 20). As demonstrated in studies of other activators, gene activation by AR is thought to require the general initiation factors that form preinitiation complexes on common core promoter elements (e.g., TATA) (44) and a variety of general and gene-specific coactivators that either modulate chromatin structure (26, 36) or serve as direct adaptors between activators and general initiation factors (43). A variety of cofactors have been implicated more directly in nuclear receptor function (32, 33, 54). On a growing list of cofactors that regulate nuclear receptors are the well-studied coactivators of p300/CBP, the p160 family (SRC-1, TIF-2/GRIP-1, ACTR/P-CIP) (54), p300/CREB-binding protein associated factor/GCN5 complexes

(yeast SAGA and human STAGA) (5, 31), and protein arginine methyltransferases (PRMTs) (48). These cofactors have histone acetyltransferase or PRMT activities and are believed to act mainly through histone acetylation or methylation and subsequent chromatin structural perturbations but can also act through functional modification of activators (21) and coactivators (10, 55). Some exhibit ligand-dependent interactions with the AF-2 domain of receptors, whereas others interact with the AF-1 domains. The multiprotein thyroid hormone receptor-associated protein (TRAP)/Mediator complexes exhibit no intrinsic histone acetyltransferase activity (30) and show subunit-specific interactions with both nuclear receptors (TRAP220 with thyroid hormone receptor [TR], vitamin D receptor, peroxisome proliferator-activated receptor, retinoic acid receptor, retinoid X receptor, and estrogen receptor [ER] and TRAP170/vitamin D receptor-interacting protein 150 with glucocorticoid receptor [GR]) and other activators (TRAP80 with p53 and VP16). This complex, in turn, interacts with the general initiation factors and polymerase II (Pol II) and acts on DNA templates at post-chromatin-remodeling steps. Of these coactivators, p300/CBP and p160s have been shown to function with AR (1, 3, 6, 23, 29), but functions of the multicomponent STAGA (~15 subunits) and TRAP (~25 subunits) complexes with AR are also likely (51). Other cofactors that have been implicated in the function of AR and, in most cases, other nuclear receptors include the ARA group, ARIP3, SNURF, FHL2, cyclin D1, and AES (22, 24). Some of these factors have broader effects on basal transcription and other activators, but less is known about their mechanistic function.

Homeoboxes are conserved 61-amino-acid DBDs present in a distinct family of transcription factors, the homeodomain

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proteins, that play a central role in eukaryotic development, with spatial and temporal specificity (19). Consistent with their role in cell growth and differentiation, homeobox gene dysfunctions have been implicated in tumorigenesis (12). NKX3.1 is a newly discovered prostate tissue-specific and androgen-regulated gene in the homeobox gene family (41). NKX3.1 is most closely related, by virtue of 78% sequence similarity with the homeodomain region, to *Drosophila* NK-3. NK-3 interacts with the corepressor Groucho through the homeodomain region to repress transcription (11). Consistent with its sequence similarity to NK-3, NKX3.1 has been shown to specifically repress transcription of a luciferase reporter containing three copies of the NKX3.1-binding site upstream of a thymidine kinase core promoter (49). The chromosomal association of the NKX3.1 gene on 8p21, a region frequently deleted in prostate cancer, suggests that NKX3.1 may function as a tumor suppressor (8). Consistent with these findings, the results of recent studies of NKX3.1 knockout mice suggest that NKX3.1 exerts a growth-suppressive effect on prostate epithelial cells and controls differentiated glandular functions (2, 7, 25, 45). These findings suggest that, as a transcription factor, NKX3.1 may play an important role in prostate cell development, cell differentiation, and tumorigenesis, even though the biological and biochemical functions of NKX3.1 remain to be deciphered.

In this study, we identified a new AR-associated protein (p44) that interacts with AR directly and enhances AR-driven gene expression *in vivo*. We also demonstrated that in the nuclei of HeLa cells, p44 forms a multiprotein complex that functions as a coactivator of AR.

## MATERIALS AND METHODS

**Establishment of prostate cell lines that stably expressed a FLAG-tagged AR or NKX3.1 and immunopurification of fAR- and fNKX3.1-associated factors.** The mammalian expression vectors pBabe-Neo-fAR and pBabe-Neo-fNKX3.1 were created by subcloning FLAG-tagged human AR or NKX3.1 cDNA into the vector pBabe-Neo. The prostate cancer cell line LNCaP was purchased from the American Type Culture Collection (ATCC; Manassas, Va.) and maintained in RPMI 1640 medium plus 10% fetal bovine serum. Cells were transfected with pBabe-Neo-fAR or pBabe-Neo-fNKX3.1 and further incubated at 37°C for 1 to 1.5 days before being split 1:6 for G418 selection (0.5 mg/ml). The medium was changed every 3 or 4 days. Individual G418-resistant colonies, normally seen after 2 weeks, were expanded into cell lines and then characterized by Western blotting using the anti-FLAG monoclonal antibody M2. The cell lines expressing FLAG-tagged AR or FLAG-tagged NKX3.1 were further expanded and analyzed. Nuclear and cytoplasmic extracts were prepared according to our standard methods (53) and used to immunopurify the AR- or NKX3.1-containing complexes. Typically, 1 ml of nuclear or cytoplasmic extract was mixed with 20  $\mu$ l of M2 resin and incubated for 3 h at 4°C with rotation. After five washes in a buffer containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM dithiothreitol, 30 mM KCl, and 0.1% NP-40, the bound proteins were eluted from the M2 agarose by incubation at 4°C for 30 min with 20  $\mu$ l of the same buffer plus 0.2 mg of the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) per ml. Aliquots (5  $\mu$ l) of eluted proteins were mixed with equal volumes of 2 $\times$  Laemmli's sample buffer and loaded onto a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel. Proteins were visualized by silver staining.

**In vitro transcription and primer extension.** The basal transcription factors TFIIA, TFIIB, TFIIE, and TFIIF and PC4 were expressed in and purified from bacteria. TFIID, TFIIH, and RNA Pol II were affinity purified from stable cells expressing the corresponding FLAG-tagged subunits (56). Transcription reactions were carried out in a final volume of 25  $\mu$ l and contained 90 fmol of supercoiled plasmid DNA template; the products were analyzed by the primer extension reaction as described previously (56).

**cDNA cloning and Northern blot analysis.** An immunopurified fNKX3.1-containing complex was subjected to SDS-polyacrylamide gel electrophoresis

(PAGE), and peptides derived from p44 were subjected to mass spectrometric analysis (37). An expression sequence tag clone (IMAGE:785275) encoding full-length p44 was obtained from the ATCC. A 1.3-kb cDNA encoding full-length p44 was labeled with  $^{32}$ P using the random primed DNA labeling kit (Boehringer Mannheim GmbH) and was used to probe human multiple-tissue Northern blot membranes (BD Biosciences Clontech, Palo Alto, Calif.).

**Expression and purification of recombinant proteins and antibody preparation.** Recombinant human AR was expressed in Sf9 cells via baculovirus vector pVL1393 as a FLAG-tagged fusion protein and purified on M2 agarose (56). His<sub>6</sub>-tagged p44 was expressed in bacteria via the expression vector pET15d and purified by affinity (nickel-nitrilotriacetic acid [Ni-NTA] agarose) and S-Sepharose chromatographic steps. The cDNA encoding amino acid residues 1 to 282 of human AR was subcloned into vector pET15d and expressed in bacteria. The His<sub>6</sub>-tagged AR(1-282) protein was purified through a Ni-NTA agarose column. Ten milligrams of the purified recombinant His<sub>6</sub>-tagged p44 and AR(1-282) proteins was sent to Conceive Inc. (Denver, Pa.) for polyclonal antibody production in rabbits. The antisera were purified through the p44 and AR(1-282) agarose columns, respectively.

**Transient transfection.** The AR, ER, GR, progesterone receptor (PR), TR, and p44 expression vectors for transfection assays were constructed by inserting their corresponding cDNA sequences into pcDNA3.1. The luciferase reporters contain the androgen, estrogen, or thyroid hormone response elements ahead of the E4 basal promoter and the luciferase gene, respectively. PC3 cells were maintained in RPMI 1640 medium plus 10% fetal bovine serum. Transfections were performed with Lipofectamine reagent (Invitrogen, Carlsbad, Calif.). Briefly, 10<sup>5</sup> cells were plated onto each well of 24-well plates approximately 24 h before transfection. After being washed with phosphate-buffered saline, cells in each well were transfected with 30 ng of an expression vector (AR, ER, GR, PR, or TR), 100 ng of the reporter plasmids, 2.5 ng of the pR-LUC internal control plasmid, and different amounts of the p44 expression vector. The total amount of DNA was adjusted to 300 ng with pcDNA3.1. Transfections were conducted in phenol-free RPMI 1640 medium; 2 h later, the medium was changed to either phenol-free RPMI 1640 plus charcoal-treated fetal bovine serum (10%) or regular medium containing 10 nM R1881, 10 nM dexamethasone, 10 nM progesterone, 1  $\mu$ M  $\beta$ -estradiol, or 10 nM T3. Cells were cultured for another 48 h and harvested for the dual luciferase assay (Promega, Madison, Wis.).

**Protein-protein interaction assay.** One microgram of recombinant glutathione S-transferase (GST) and GST fusion proteins (GST-p44, GST-NTD, GST-DBD, and GST-LBD) were expressed in bacteria and immobilized on 20  $\mu$ l of glutathione-Sepharose beads. The beads were incubated with 5  $\mu$ l of rabbit reticulocyte lysate containing  $^{35}$ S-labeled AR, NKX3.1, or PRMT5 in a final volume of 200  $\mu$ l containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM dithiothreitol, 150 or 300 mM KCl, and 0.1% NP-40. The beads were washed five times (1 ml each) with the incubation buffer, boiled in 20  $\mu$ l of the SDS gel sample buffer, and analyzed by SDS-PAGE followed by autoradiography.

**ChIP.** LNCaP cells were grown in phenol red-free RPMI 1640 supplemented with charcoal-dextran-stripped fetal bovine serum (10%) for 2 days and then treated with 1 nM R1881 for 16 h. Cells treated with ethanol were used as the control. Chromatin immunoprecipitation (ChIP) was performed as described previously (39) with the following modifications. Cells were cross-linked with 1% formaldehyde at room temperature for 10 min and the cross-linking reaction was stopped by addition of glycine to 0.125 M. The cross-linked chromatin was sonicated with a Branson Sonifier 450 microtip at power setting 6 for five 30-s bursts separated by cooling on ice. This treatment produced DNA fragments of average size of 700 bp. For immunoprecipitation, 2  $\mu$ g of antigen-purified anti-AR or anti-p44 antibody was mixed with 300  $\mu$ g of the purified cross-linked chromatin and incubated overnight at 4°C. Immunocomplexes were washed five times (10 min each) in 1 ml of the buffer containing 1% Triton X-100, 0.1% sodium deoxycholate, 0.05% SDS, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride; once in a solution containing 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0; and twice in 1 mM EDTA–10 mM Tris-HCl, pH 8.0. After reversal and recovery of the immunoprecipitated chromatin DNA, the final DNA pellets were dissolved with 50  $\mu$ l of H<sub>2</sub>O. Immunopurified DNA (5  $\mu$ l) was used for a PCR (30 cycles, annealing at 50°C), with primers as follows. For prostate-specific antigen (PSA), the forward primer sequence was TCTGCTTTGTCCGCTAGAT and the reverse primer sequence was AACCTTCATCCCCAGGACT, which amplifies a 212-bp product from –250 to –39 upstream of the PSA transcription start site. For  $\beta$ -actin, the forward primer sequence was TCCTCTCTTCTCAATCTCG and the reverse primer sequence was AAGGCAACTTTCGGAACGG, which amplifies a 145-bp product from –118 to –974 of the  $\beta$ -actin gene (the A of the ATG translation start codon was arbitrarily given the number +1).



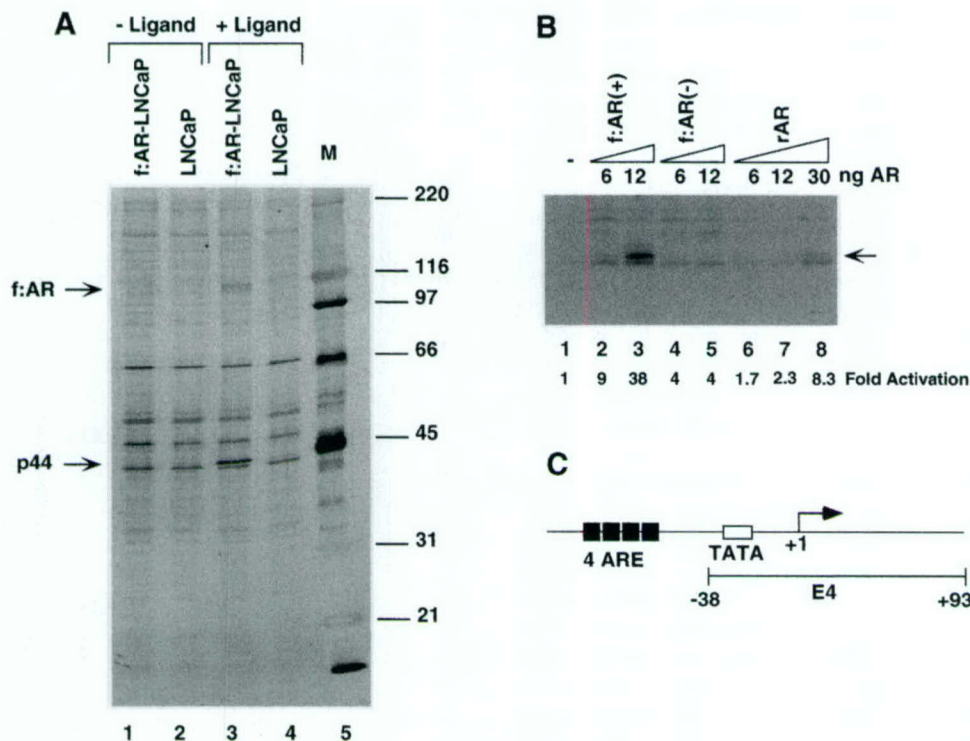


FIG. 1. Effects of affinity-purified f:AR-cofactor complexes on transcription in a system reconstituted with purified factors. (A) SDS-PAGE analysis of f:AR-cofactor complexes. Lanes 1 and 3 show AR-containing complexes immunopurified from nuclear extracts made from a stably transfected, FLAG-tagged, AR-expressing LNCaP cell line grown in the presence (lane 3) or absence (lane 1) of the synthetic androgen R1881 (10 nM). The gel was stained with silver. Bands corresponding to FLAG-tagged AR (f:AR) and to the polypeptide specifically associated with AR are indicated by arrows. (B) AR- and AR-cofactor-dependent transcription. A synthetic template containing four ARE elements (C) was transcribed in the system reconstituted with purified factors (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, Pol II, and PC4) with additions of the rAR and f:AR-cofactor complexes described for panel A. The specifically initiated transcript is indicated by an arrow and was monitored by primer extension. The activation relative to levels of transcription in the absence of rAR or f:AR complexes (lane 1) is indicated at the bottom. (C) Diagram of the synthetic ARE-containing template. The template (pARE-E4) contains four tandem copies of the ARE from the PSA promoter positioned upstream of the adenovirus E4 promoter.

**Methylation of proteins.** A cDNA (IMAGE:3833019) encoding the full-length human PRMT5 was purchased from the ATCC. The point mutant (R368A) was created by using a QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. The mutation was confirmed by DNA sequencing analysis. The wild-type and mutant PRMT5 were expressed in bacteria via pET15d expression vector and purified through Ni<sup>2+</sup>-NTA agarose. The methylation assay was performed as follows. Two micrograms of the purified histones (27) was incubated with 0.8  $\mu$ g of the purified recombinant wild-type or mutant PRMT5 and various factors in 25  $\mu$ l of 50 mM Tris, pH 7.5–1 mM EDTA–1 mM EGTA–20  $\mu$ M of [<sup>3</sup>H]AdoMet (Amersham Pharmacia Biotech) at 30°C for 30 min. The reactions were stopped by the addition of 5  $\mu$ l of 5 $\times$  SDS sample loading buffer, and samples were resolved by SDS–15% PAGE. The gels were stained with Coomassie blue R250, destained, treated with an intensifying solution, and analyzed by autoradiography.

**In situ hybridization.** We used matched normal and cancerous prostate tissues derived from radical prostatectomies of patients with prostate cancer at New York University Medical Center in an institutional review board-approved protocol. The procedure for in situ hybridization was as described previously (28). Briefly, the sections were hydrated, postfixed in 4% paraformaldehyde, and treated with proteinase K followed by deacetylation. The prehybridization and hybridization treatments were performed at 68°C using 0.3 M NaCl and 50% formamide. DNA fragments (500 bp, cDNA sequences of p44 from 1 to 500) containing both T7 and T3 promoters were generated by PCR. Corresponding <sup>32</sup>P-labeled RNAs (sense and antisense) were generated by in vitro transcription with T7 and T3 RNA polymerases, respectively, and hybridized to the tissue sections (4  $\mu$ m). After being washed, the slides were exposed to NTB-2 X-ray emulsion (Eastman-Kodak, Rochester, N.Y.) for 2 to 3 weeks and counterstained with hematoxylin-eosin. Image and statistical analyses were performed as described previously (28).

## RESULTS

**Immunopurification and functional analysis of FLAG-AR-associated factors.** Stable cell lines expressing FLAG-epitope-tagged factors were generated previously and used to affinity purify corresponding parental complexes (52, 53, 56). To similarly identify androgen-dependent or androgen-independent AR-interacting factors, we generated a prostate cancer cell line (f:AR-LNCaP) that stably expresses a FLAG-tagged AR. Immunopurification of f:AR from nuclear extracts made from f:AR-LNCaP cells grown in the presence and absence of androgen (R1881) revealed androgen (R1881)-induced association of a 44-kDa f:AR-associated polypeptide (Fig. 1A, lane 3). The specific association of this polypeptide with f:AR is further shown by the failure of similar-sized polypeptides in extracts from control cells (not expressing f:AR) to bind to the affinity matrix (Fig. 1A, lane 4).

When assayed in the purified minimal transcription system containing TFIIA, TFIIB, TFIID, TFIIF, Pol II, and PC4 with the synthetic ARE-containing template (56) (Fig. 1C), recombinant AR (rAR) alone elicited up to 8.3-fold activation (Fig. 1B, lanes 6 to 8). The f:AR complex from nuclear extract derived from f:AR-expressing LNCaP cells grown in the presence of R1881 [f:AR(+)] elicited up to 38-fold activation (Fig.



1B, lanes 2 and 3) and at an equimolar input was 16-fold more active than rAR (Fig. 1B, lane 3 versus lane 7). In contrast, the f:AR complex from nuclear extract of f:AR-expressing LNCaP cells grown in the absence of R1881 [f:AR(-)] showed a level of activity only about 2-fold above that shown by rAR (Fig. 1B, lanes 4 and 5 versus lanes 6 and 7). The amounts (indicated in Fig. 1B) of recombinant AR and f:AR in the f:AR-containing complexes were normalized by quantitative Western blot analysis with anti-AR polyclonal antibodies. These results indicate that polypeptides associated with AR specifically in the presence of androgen can upregulate AR function; a likely candidate is the 44 kDa protein, although other minor polypeptides could also be responsible. When the cell line was further expanded, the 44-kDa polypeptide became less abundant, and an attempt to isolate a sufficient amount of the 44-kDa polypeptide for peptide sequence analysis failed.

**Purification, cloning, and characterization of FLAG-NKX3.1-interacting proteins.** We similarly generated a prostate cancer cell line (f:NKX3.1-LNCaP) that stably expresses a FLAG-tagged NKX3.1. Immunoprecipitation of f:NKX3.1 from extracts isolated from f:NKX3.1-LNCaP cells revealed 44- and 170-kDa polypeptides that specifically associate with NKX3.1 in the cytoplasm (Fig. 2A, lane 5), but not with NKX3.1 in the nucleus (Fig. 2A, lane 3). The specific association of these polypeptides with f:NKX3.1 is further shown by the failure of similar-sized polypeptides in extracts from control cells (expressing f:SRC1) to bind to the affinity matrix (Fig. 2A, lane 4). Because f:NKX3.1 stained negatively with silver, Western blot analysis with the anti-FLAG antibody was employed to demonstrate the existence of f:NKX3.1 in immunoprecipitates derived from both nuclear (Fig. 2A, bottom panel, lane 3) and cytoplasmic (Fig. 2A, bottom panel, lane 5) extracts. After larger amounts of f:NKX3.1-associated 44-kDa protein had accumulated, we performed direct sequence analysis by mass-spectrometric methods (37). On the basis of the peptide sequence (KETPPPLVPPAAR) obtained from mass-spectrometric analysis, we obtained a cDNA encoding the 44-kDa protein. The p44 cDNA encodes a protein containing 342 amino acid residues and four putative WD-40 repeats (residues 68 to 107, 114 to 153, 157 to 196, and 280 to 319). p44 is identical in sequence to the recently identified MEP50 component of the methylosome (18) and to the WD45 subunit of the survival motor neuron (SMN) complex (35). Northern blot analysis of multiple human tissues showed that p44 mRNA is highly expressed in the heart, skeletal muscle, spleen, testis, uterus, prostate, and thymus (Fig. 3). Western blot analysis with anti-p44 antibody revealed that the f:AR preparation immunopurified from the f:AR-LNCaP cell line (Fig. 1A, lane 3) contains the same 44-kDa polypeptide (Fig. 2B, lane 3).

**p44 specifically enhances AR-dependent transcription in vivo.** To investigate the effect of p44 on AR-dependent transcription in vivo, a luciferase reporter (4× ARE-E4-Luc) containing four tandem PSA promoter AREs (-152 to -174) (14) and the minimal adenovirus E4 promoter (-38 to +93) upstream of the luciferase gene was cotransfected with expression vectors for AR and p44 into PC3 cells in the absence or presence of ligand (R1881). As shown in Fig. 4A, AR activated the reporter gene about 12-fold in the presence of ligand, and coexpressed p44 showed a strong (up to 3.3-fold) enhancement of this activity that is likely restricted in magnitude by contri-

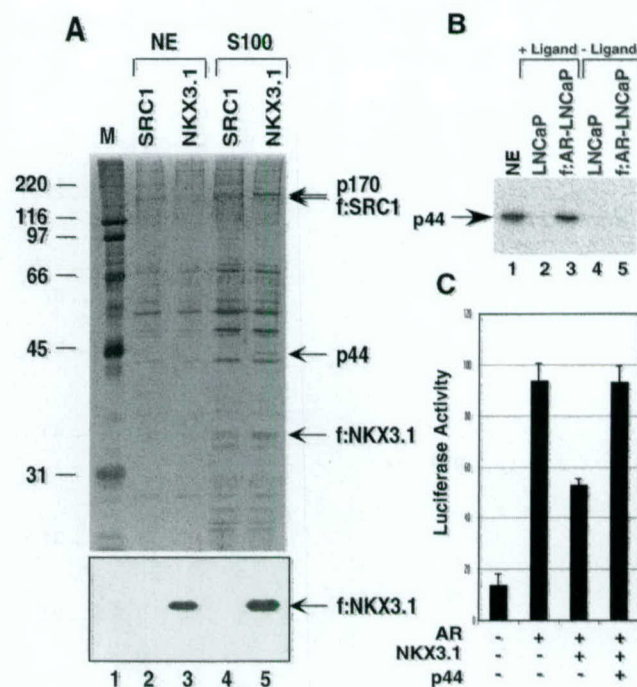


FIG. 2. p44 associates with NKX3.1 in the cytoplasm. (A) SDS-PAGE analysis of purified f:NKX3.1-containing complex. Immunoprecipitation was performed with nuclear extracts (NE) (lane 3) and cytoplasmic extracts (lane 5) made from a stably transfected, FLAG-tagged, NKX3.1-expressing cell line. Bands corresponding to FLAG-tagged NKX3.1 and polypeptides specifically associated with NKX3.1 (p44 and p170) are indicated by arrows. The specific association of these polypeptides with f:NKX3.1 is further shown by the failure of similarly sized polypeptides in extracts from control cells (expressing f:SRC1) to bind to the affinity matrix (lanes 2 and 4). Lane 1, standard molecular weight markers (Bio-Rad). The bottom panel is a Western blot of the same samples with anti-FLAG monoclonal antibody. (B) Western blot analysis of the f:AR-complexes with anti-p44 antibody. Lane 1 contains 5  $\mu$ l of nuclear extract made from LNCaP cells. (C) NKX3.1 partially represses AR-dependent gene expression, and the overexpression of p44 relieves this repression. PC3 cells were transfected with 100 ng of 4× ARE-E4-luc reporter plasmid, 30 ng of pcDNA-AR, 60 ng of pcDNA NKX3.1, and 100 ng of p44, as indicated. Cells were grown in the presence of 10 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays.

butions from endogenous p44 (data not shown). p44 did not influence reporter gene activity in the absence of cotransfected AR or ligand (R1881), indicating that the enhancing effect of p44 on AR-dependent gene expression was caused by an effect on the E4 promoter. To investigate the receptor specificity of p44, we examined the effects of p44 on transcription of reporters containing the same E4 core promoter under the control of GR, PR $\beta$ , ER $\alpha$ , and TR. As shown in Fig. 4A, p44 also enhanced GR- and PR-driven gene expression and, in contrast, showed no effect on TR- or ER-mediated transcription. Hence, p44 shows some nuclear receptor-specific effects in vivo. p44 also enhanced AR-driven transcription from natural ARE-containing mouse mammary tumor virus (15) and probasin (-244 to +12) (13) promoters but had no obvious effects on the promoter derived from the PSA enhancer (-4354 to -3858) (46) (Fig. 4B). These results suggest that p44 has promoter specificity.



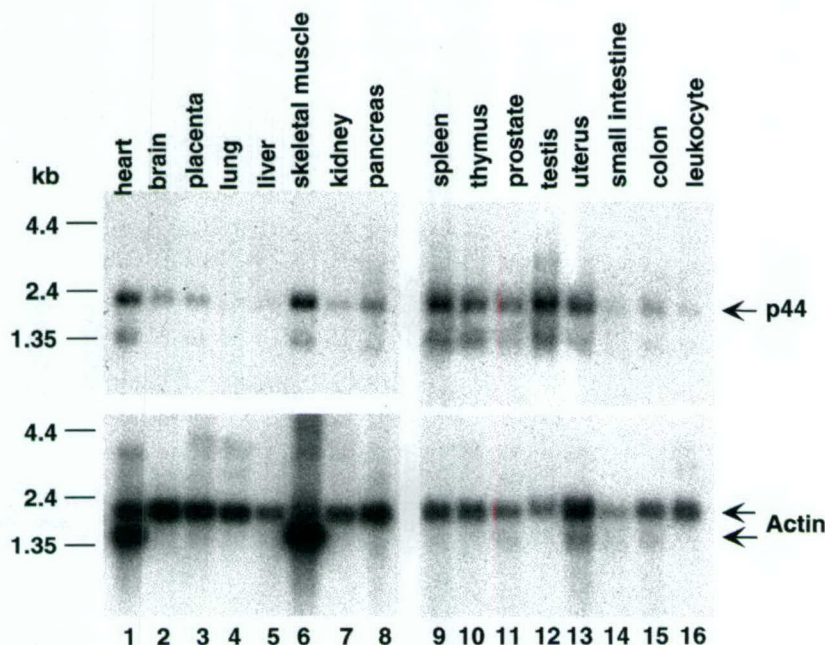


FIG. 3. Northern blot analysis of p44 expression. The membranes were probed with  $^{32}\text{P}$ -labeled p44 cDNA (top) and  $^{32}\text{P}$ -labeled  $\beta$ -actin cDNA (bottom).

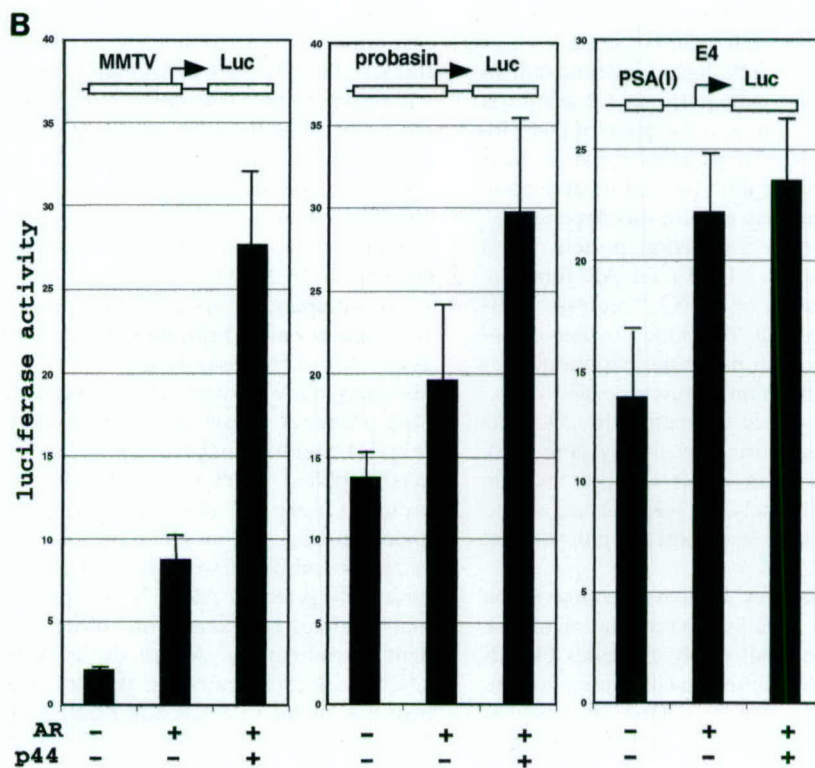
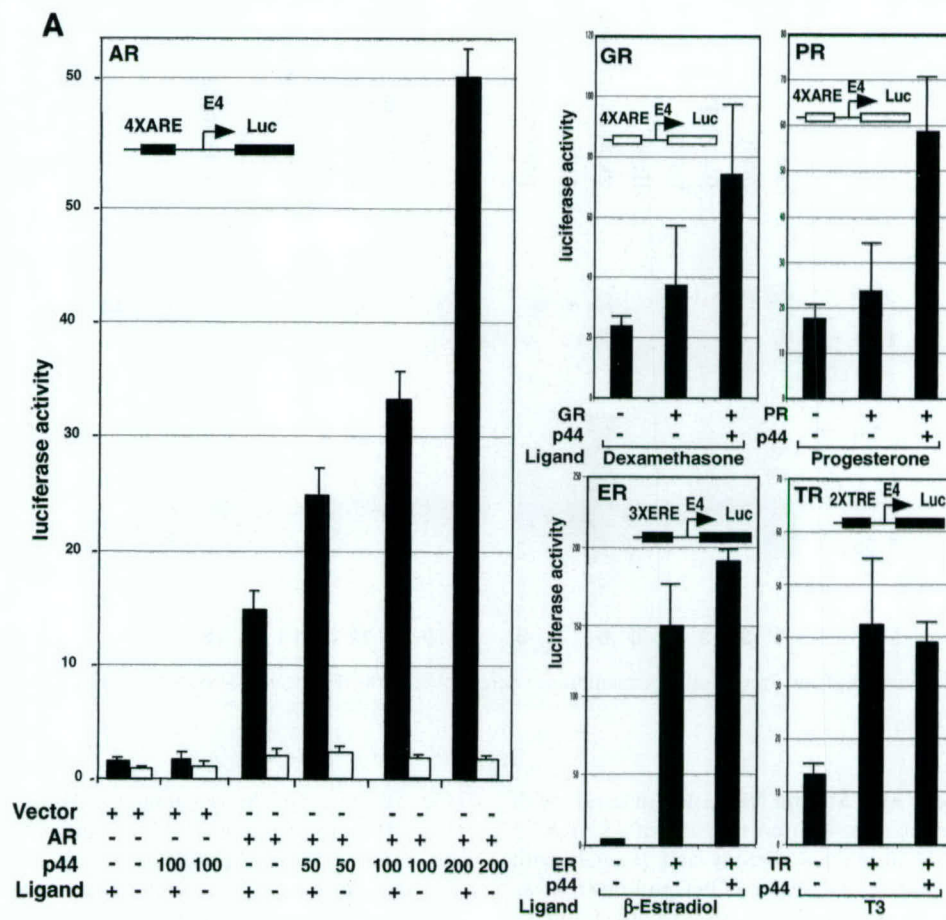
**p44 interacts directly with AR and NKX3.1.** Consistent with the observed intracellular association of p44 with f:NKX3.1 (Fig. 2A, lane 5), p44 interacted directly and strongly with NKX3.1 in a salt-insensitive manner in a GST pull-down assay (Fig. 5A, lanes 10 to 14). Recombinant AR was also found to interact strongly (but in a salt-sensitive manner) with a GST-p44 fusion protein but not with GST alone (Fig. 5A, lanes 1 to 9), which was consistent with the intracellular association of f:AR with p44 (Fig. 1A, lane 3). The ligand-independent *in vitro* interaction of purified p44 with purified AR contrasts with the ligand-dependent intracellular association of p44 with AR. However, other AR-associated proteins (such as heat shock proteins) whose interactions are reversed by androgen, thus facilitating p44 interaction, may explain this dependency. Our identification of a common interacting protein (p44) prompted studies of the effect of NKX3.1 on AR function, which showed that overexpression of NKX3.1 represses AR-driven gene expression *in vivo* (Fig. 2C). One possible explanation is that NKX3.1 may sequester p44 in the cytoplasm, thus inhibiting AR-driven gene expression. Overexpression of ectopic p44 was shown to completely overcome the NKX3.1-mediated repression, which supports this theory (Fig. 2C). Since p44 enhances AR activity in either the presence (in LNCaP cells) or absence (in PC3 cells) of NKX3.1, the mechanism of enhancement by p44 may be completely independent of NKX3.1 action.

**p44-containing complex enhances AR-driven transcription *in vitro*.** To further explore the function and regulation of p44, we established a HeLa cell line that stably expresses FLAG-tagged p44 and used it to immunopurify p44-containing complexes. SDS-PAGE analysis revealed a large number of polypeptides (more abundant in the 50- to 60-kDa range and less abundant in the 70- to 100-kDa and 10- to 30-kDa ranges)

(Fig. 5B, lane 5). The recombinant p44 expressed in bacteria (Fig. 5B, lane 2) inhibited AR-dependent transcription from the synthetic ARE-E4 promoter (Fig. 5C, lane 3 versus lane 2). This repression contrasted with the activation observed *in vivo* (transient transfection) (Fig. 4). One possible reason for this is that p44 activation of AR-driven gene expression requires additional factors. In the absence of these factors in the *in vitro*-reconstituted transcription system, p44 might sequester some transcription factors (such as AR) through direct interactions and thus repress transcription. This hypothesis is supported by our finding that the p44-containing complex purified from the FLAG-tagged stable cell line enhanced AR-driven transcription from the same promoter (Fig. 5C, lane 5). The p44-containing complex did not affect GAL4-VP16-driven transcription in the same *in vitro* transcription system (data not shown).

The occupancy of specific DNA sites by specific DBDs (e.g., AR) and associated proteins can be established by the ChIP assay (38). This assay is a direct and powerful method of assessing *in vivo* protein-DNA interactions. AR binds to the PSA promoter region in the presence of androgen (R1881) (Fig. 5D, middle panel, lane 1). As negative controls, the products amplified by PCR at the same time from the  $\beta$ -actin promoter were not changed in response to the addition of androgen (Fig. 5D, middle panel, lanes 3 and 4). This observation is consistent with the fact that the PSA promoter is directly targeted by AR (57). We performed this assay more than 10 times and consistently observed the androgen-dependent recruitment of AR to the PSA proximal promoter in LNCaP cells. Cofactors can also be cross-linked by formaldehyde treatment to chromatin through their interactions with DNA-binding factors in living cells. Therefore, the ChIP assay is also a direct way to determine cofactor occupancy on AR







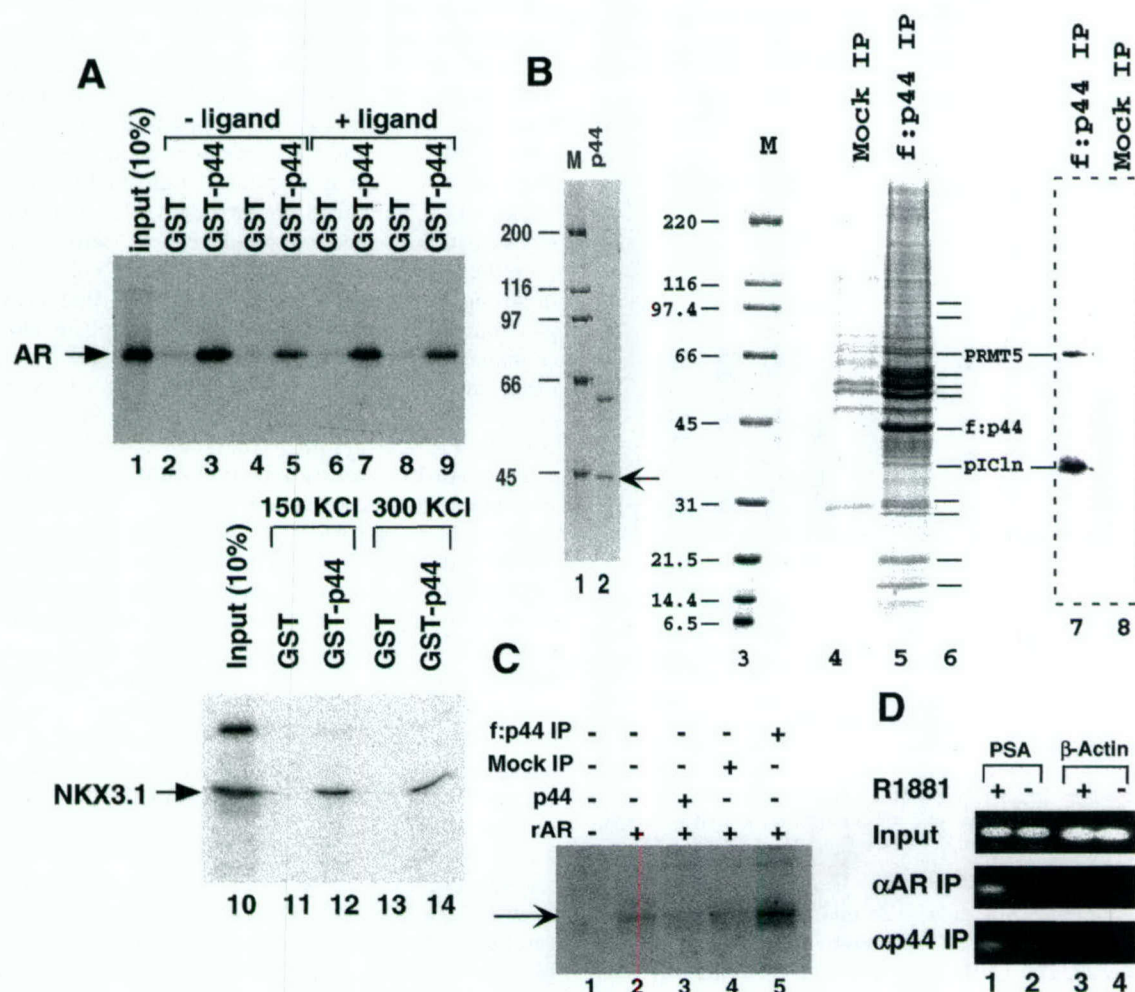


FIG. 5. p44-containing complex enhances AR-driven transcription. (A) p44 interacts directly with AR and NKX3.1. GST-p44 fusion protein expressed in bacteria was immobilized on glutathione agarose beads. Beads were incubated with  $^{35}$ S-labeled AR (lanes 1 to 9) or NKX3.1 (lanes 10 to 14) in BC150–0.1% NP-40 (lanes 2, 3, 6, 7, 11, and 12) or BC300–0.1% NP-40 (lanes 4, 5, 8, 9, 13, and 14) in the absence (lanes 2 to 5 and 10 to 14) or presence (lanes 6 to 9) of 50 nM R1881 for 2 h at 4°C. After being washed with the incubation buffer, the beads were boiled with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography. (B) SDS-PAGE analysis of purified p44 and p44-containing complexes. Lane 2 shows recombinant p44 expressed in bacteria and purified on an Ni-NTA agarose affinity column; lanes 5 and 4 show p44-containing complexes immunopurified from nuclear extracts made from a stably transfected, FLAG-tagged, p44-expressing HeLa cell line and immunoprecipitate from extracts made from control cells (not expressing f:p44), respectively. The gels were stained with Coomassie blue R250. The band corresponding to p44 is indicated by the arrow at the right. Polypeptides specifically associated with p44 are indicated by short lines at the right (lane 6). Lanes 1 and 3, standard molecular weight markers (Bio-Rad); lanes 7 and 8, Western blot analysis of the immunoprecipitate isolated from p44-expressing cells (lane 7) and control cells (lane 8) using anti-PRMT5 and anti-pICln antibodies. (C) The p44-containing complex enhances AR-dependent transcription. A synthetic template, pARE-E4, was transcribed in the system reconstituted with purified factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, Pol II, and PC4) with additions of rAR, p44, and the f:p44-containing complex described for panel B. The specifically initiated transcript is indicated by an arrow and was monitored by primer extension. (D) p44 was recruited on the PSA promoter in the presence of the androgen. LNCaP cells were grown in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of 1 nM R1881. A ChIP assay was performed with antigen-purified anti-AR (middle panel) or anti-p44 (top panel) antibodies. The purified-protein–DNA cross-links were reversed, and the resulting DNA was amplified by a PCR with two specific primers derived from promoter regions of PSA (lanes 1 and 2) or  $\beta$ -actin (lanes 3 and 4). The same set of PCRs (top panel) was performed with chromatin DNA (Input) used for the ChIP assay.

FIG. 4. p44 specifically enhances AR-mediated transcription in vivo. (A) p44 enhanced AR-, GR-, and PR-mediated transcription. PC3 cells were transfected with 100 ng of 4 $\times$  ARE-, 3 $\times$  ERE-, or 2 $\times$  TRE-E4-luc reporter plasmid, 30 ng of pcDNA-AR, -GR, -PR, -ER, or -TR, and the indicated amounts of pcDNA-p44 expression plasmid. Cells were grown in the absence or presence of 10 nM R1881, 10 nM dexamethasone, 10 nM progesterone, 1  $\mu$ M estradiol, or 10 nM T3 for 48 h after transfection and then harvested for luciferase activity assays. (B) p44 selectively affected AR-mediated luciferase gene expression from different promoters. PC3 cells were transfected with 100 ng of MMTV-, probasin-, or PSA(I)-luc reporter plasmid, 30 ng of pcDNA-AR, and 150 ng of pcDNA-p44 expression plasmid. Cells were grown in the presence of 10 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays.



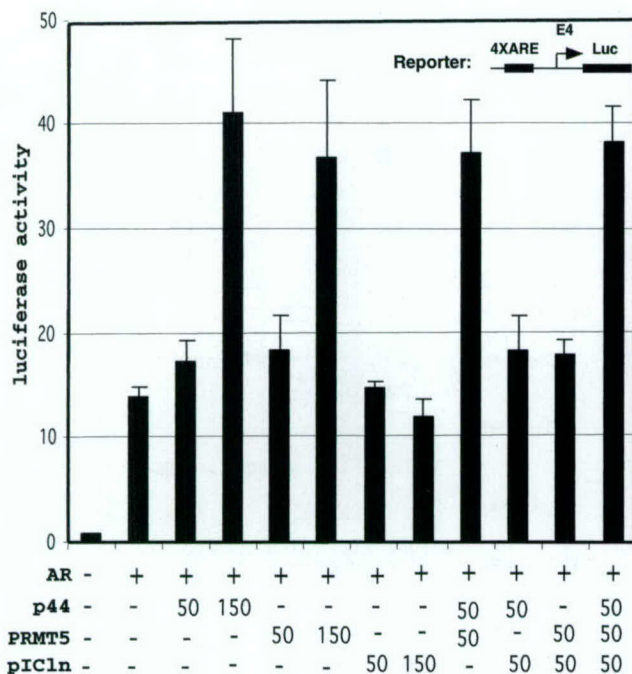


FIG. 6. PRMT5 synergizes with p44 to enhance AR-driven gene expression. PC3 cells were transfected with 100 ng of ARE-E4-luc reporter plasmid, 30 ng of pcDNA-AR, and indicated amounts (in nanograms) of pcDNA-p44, -PRMT5, or -pICln expression plasmid. Cells were grown in the absence or presence of 10 nM R1881 for 48 h after transfection and then harvested for dual luciferase activity assays.

target genes. Figure 5D shows the androgen-dependent recruitment of p44 onto the PSA promoter (bottom panel, lane 1 versus lane 2). The ChIP assay with anti-p44 antibody was independently performed twice, and the results were consistent. We found that when larger amounts (twofold) of DNA were used in our standard PCRs (30 cycles), we still observed the androgen-dependent recruitment of p44 to the PSA promoter, although the background was slightly higher. However, fewer amplification cycles (27 cycles) in the PCR gave better results when the larger amounts of DNA were used. These results suggest that p44 functions on the AR target gene in vivo.

Others have reported that MEP50 and WD45 form complexes with PRMT5 and pICln in the methylosome and SMN complexes, respectively (18, 35). To further establish whether the latter two proteins are present in our p44-containing complex, Western blot analysis with anti-PRMT5 and anti-pICln antibodies was performed. Both PRMT5 and pICln proteins were present in the p44-containing complex (Fig. 5B, lane 7). To determine whether PRMT5 and pICln are involved in AR-driven gene expression, we subcloned cDNAs encoding PRMT5 and pICln (IMAGE:3836445) (ATCC) into the expression vector pcDNA3.1. To investigate the effect of PRMT5 and pICln on AR-dependent transcription in vivo, an ARE-containing luciferase reporter was cotransfected with expression vectors for AR, PRMT5, pICln, or different combinations into prostate cancer PC3 cells in the presence of ligand (R1881). As shown in Fig. 6, AR activated the reporter gene about 12-fold, and coexpressed PRMT5 showed a strong (up to

2.5-fold) enhancement of this activity. PRMT5 did not influence reporter gene activity in the absence of cotransfected AR or ligand (R1881) (data not shown), indicating that the enhancing effect of PRMT5 on AR-dependent gene expression was caused by an effect on the E4 promoter. To investigate the effect of PRMT5 plus p44 in the same assay, we cotransfected PC3 cells with limited amounts (50 ng) of PRMT5 and p44 alone or in combination. Figure 6 shows that 50 ng of PRMT5 or p44 had little effect on AR-dependent transcription. However, the same amounts of combined PRMT5 plus p44 resulted in strong (threefold) activation, indicating that PRMT5 and p44 function synergistically. In contrast, pICln alone or in combination with p44, PRMT5, or both had no significant effect on AR-dependent transcription (Fig. 6). Western blot analysis indicated that f:AR complex (Fig. 1A, lane 3) also contains PRMT5 and pICln (data not shown).

**The methyltransferase activity of PRMT5 is not required for the enhanced transactivation of AR.** We further studied the interactions among AR, p44, and PRMT5. In vitro-produced, <sup>35</sup>S-labeled PRMT5 was incubated with immobilized GST, GST-p44, GST-NTD, GST-DBD, and GST-LBD. After being washed, bound proteins were resolved by SDS-PAGE and visualized by autoradiography. p44 directly interacted with PRMT5 (Fig. 7A, lane 3). In contrast, PRMT5 did not bind any domain (NTD, DBD, or LBD) of AR (Fig. 7A, lanes 7 to 9). These results indicate that PRMT5 is recruited to AR target genes through its direct interaction with p44. The conserved arginine residue (R368) is essential for the methyltransferase activity of PRMT5 (40). The wild-type (Fig. 7B, lanes 2 and 3) and R368A mutant (lanes 4 and 5) PRMT5 were expressed in and purified from bacteria. Incubations of histones purified from HeLa cells with [<sup>3</sup>H]AdoMet plus recombinant PRMT5 resulted in transfer of the radioactive methyl groups to the histone H4 (Fig. 7C, top panel, lane 2). In contrast to previous observations (40), we did not detect the methylation of the histone H2A by PRMT5. This discrepancy might be due to the different preparations of histones used for the methylation assay. The preparation of individual histones was used in the previous study, but purified natural histones (containing the octamer of 2H2A, 2H2B, 2H3, and 2H4) were used in our study. The R368A mutation on PRMT5 dramatically reduced the methyltransferase activity in vitro (Fig. 7C, top panel, lane 3 versus lane 2). However, this mutation did not significantly decrease the PRMT5-mediated transactivation of AR in vivo (Fig. 7D), indicating that the methyltransferase activity of PRMT5 is not required. Western blot analysis with anti-PRMT5 antibody revealed that the levels of the wild-type and mutant PRMT5 in the transfected PC3 cells are same (data not shown). However, the same mutation on PRMT5 impaired its activity as transcriptional corepressor on the cyclin E1 promoter (16).

**Overexpression of p44 correlates with prostate tumorigenesis.** In order to investigate the possibility that p44 might be important for modulating AR function in prostate cancer, we investigated the expression of p44 at the mRNA level using quantitative in situ hybridization methods in 43 primary prostate cancers with different degrees of differentiation. Expression of p44 was detected only in prostate epithelial cells and was significantly up-regulated in 36% of well-differentiated prostate tumors (Table 1; Fig. 8a, b, g, and h), in more than



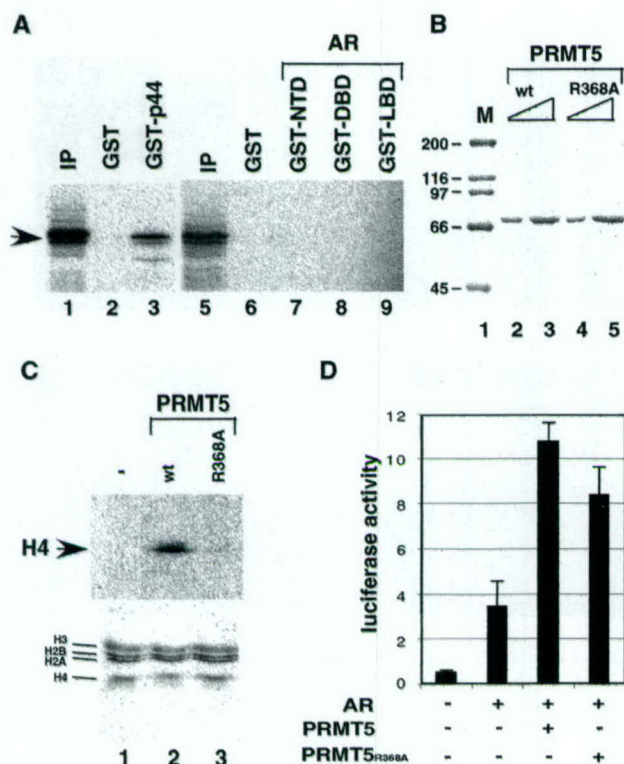


FIG. 7. (A) p44 physically interacted with PRMT5. The <sup>35</sup>S-labeled PRMT5 were incubated with the indicated GST fusion proteins. After being washed, bound proteins were resolved by SDS-10% PAGE and visualized by autoradiography. Lanes 1 and 5 contain 10% of the labeled PRMT5 used in binding reactions. (B) SDS-PAGE analysis of recombinant wild-type (lanes 2 and 3) and R368A mutant (lanes 4 and 5) PRMT5 expressed in bacteria and purified on a Ni-NTA agarose affinity column. The gel was stained with Coomassie blue R250. Lane 1, standard molecular weight markers (M) (Bio-Rad). (C) In vitro methyltransferase assay. The methyltransferase assay was performed as described in Materials and Methods. (Top) Autoradiography of the gel; (bottom) Coomassie blue staining of the same gel. Individual histones are indicated on the left. (D) Methyltransferase activity is not required for PRMT5 function on AR-driven gene expression. PC3 cells were transfected with 100 ng of 4× ARE-E4-luc reporter plasmid, 30 ng of pcDNA-AR, and 150 ng of pcDNA-PRMT5 or pcDNA-PRMT5(R368A) expression plasmid, as indicated. Cells were grown in the presence of 10 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays.

80% of moderately differentiated prostate tumors (Table 1; Fig. 8c, d, i, and j), and in more than 60% of poorly differentiated prostate tumors (Table 1; Fig. 8e, f, k, and l). The average increase was 5.1-fold. These results indicate that changes in p44 expression (and possibly in expression of associated proteins) might play an important role in affecting the

TABLE 1. Quantitative data for the in situ hybridization analysis

Tumor grade	No. of cases	No. with increase in p44 <sup>a</sup>	%
Low	11	4	36
Moderate	23	19	82
High	10	6	60

<sup>a</sup> Number of tumors with a 2- to 10-fold increase in p44 expression.

deregulation of normal AR and NKX3.1 functions in prostate tumorigenesis.

## DISCUSSION

In this study, we describe the isolation of p44 as a new AR- and NKX3.1-interacting protein both in vitro and in vivo. Transient-transfection assays demonstrated that p44 increased AR transcriptional activity in an androgen-dependent manner. p44 forms a multiprotein complex that enhanced AR-dependent transcription in a cell-free transcriptional system.

**A novel cofactor complex functions as an AR coactivator.** Increasing numbers of cofactors have been indicated in the function of AR (22, 24). They are identified through their physical interactions with AR and enhanced or repressed AR-mediated transcription in vivo. Our attempt to isolate the AR-associated proteins from stable cell lines resulted in the identification of p44. p44 and p44-containing complex enhanced AR-dependent transcription in vivo and in vitro, respectively. The protein sequence of p44 is identical to that of a component (MEP50) of the methylosome (18) and a subunit (WD45) of the SMN complex (34). The methylosome complex contains PRMT5/JBP1, pICln, and Sm proteins and mediates the assembly of spliceosomal snRNP (17). MEP50 is important for methylosome activity and binds to PRMT5/JBP1 and to a subset of Sm proteins (18). SMN is part of a complex that contains the Sm proteins and PRMT5 and is necessary and sufficient for assembly of spliceosomal U-rich snRNP (35, 47). The methylosome and SMN complexes were isolated from the cytoplasm of HeLa cells, and the p44-containing complex was purified from HeLa cell nuclear extract (17, 35). Thus, p44 may form distinct complexes with different proteins in the cytoplasm and in the nucleus for different roles (transcription versus splicing and/or translocation). The apparent size of MEP50 (above that of the 45-kDa bovine serum albumin) revealed by SDS-PAGE (17, 18) is larger than that of p44 and WD45 (below that of the 45-kDa bovine serum albumin), indicating that posttranslational modifications may exist in MEP50.

**PRMT5 is present within the p44-containing complex.** Two types of PRMT activities have been identified in mammalian cells (58). PRMT1, PRMT2, and PRMT4/CARM1 have been found to participate in nuclear receptor transcriptional activation (42, 48, 50). The methylation of histones H3 and H4 by PRMT1 and PRMT4/CARM1 correlates with transcriptional activation, suggesting that they act by modifying chromatin structure. More recently, PRMT5 was identified as a corepressor of cyclin E1 transcription (16). Forced expression of PRMT5 negatively affected cyclin E1 promoter activity, which required the methyltransferase activity of PRMT5 (16). In contrast, our results demonstrate that PRMT5 is a positive AR cofactor that functions in a methyltransferase activity-independent manner in transient-transfection assays. Since the reporter gene in the transient transfection is likely not well packaged into chromatin, we cannot rule out the involvement of the methyltransferase activity of PRMT5 in AR function on the genes integrated into chromatin. Similarly, PRMT2 was identified as a methyltransferase based on the protein sequence and functioned as a positive cofactor for ERα, but so far its methyltransferase activity has not been identified with substrates including histones and ERα (42). The enhancement of



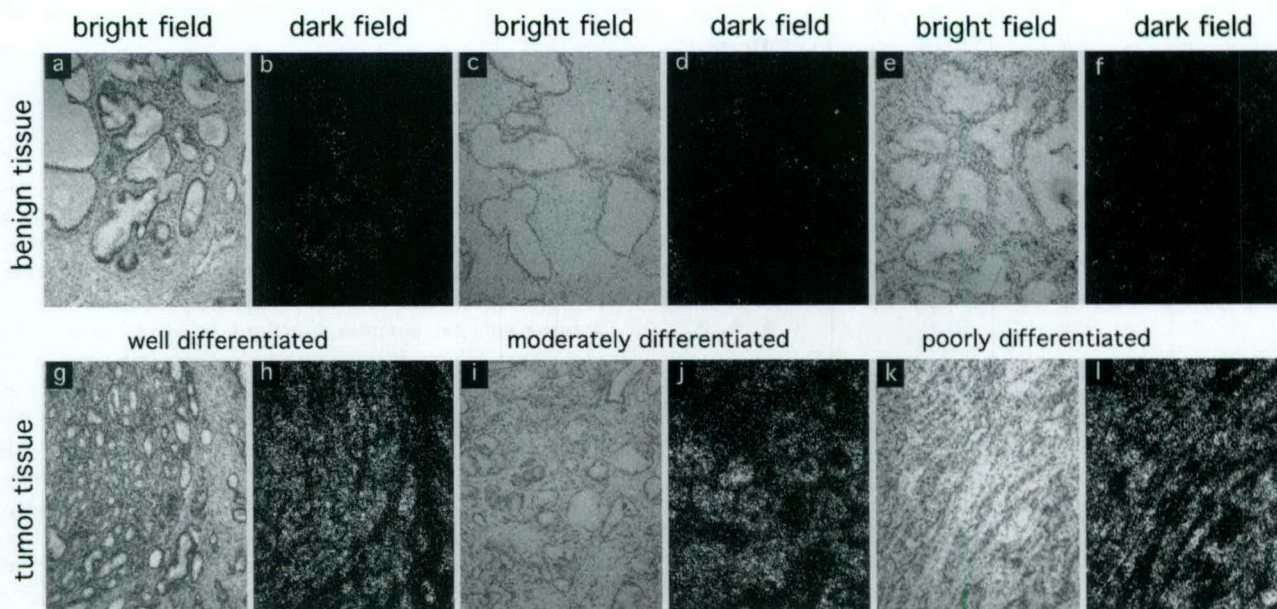


FIG. 8. Enhanced expression of p44 in prostate tumor tissues. Frozen sections (4  $\mu$ m thick) of prostate tissues were prepared and kept frozen until used. The frozen tissue sections were fixed in 4% paraformaldehyde for 30 min, dehydrated with ethanol, and hybridized with antisense p44 RNA probes labeled in vitro with [ $\alpha$ - $^{32}$ P]UTP. The slides were washed first with  $2\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and then twice with  $0.2\times$  SSC at 45°C for 20 min. The slides were exposed and evaluated with a Nikon microscope with a digital camera interfaced to a computer.

AR-dependent transcription by PRMT5 might result from activation domains existing within PRMT5 protein or from the structural role of PRMT5 required for assembling the p44-containing cofactor complex. The former possibility is not supported by that fact that no activation was observed when PRMT5 was tethered to DNA through the DBD of GAL4 (data not shown).

**p44 is overexpressed in prostate cancer.** The observation that certain cofactors are abnormally expressed in some prostate cancers indicates the importance of nuclear receptor cofactors in transcriptional control of AR function and also points to their possible role in neoplastic conversion (28). Overexpression of p44 in prostate cancer tissues indicates that it may play an important role in prostate tumorigenesis, and there is well documented evidence that abnormal NKX3.1 expression is involved in prostate tumorigenesis. Our finding that p44 interacts with both NKX3.1 and AR suggests that it might play a role in coregulating these two pathways.

In summary, our results point to a novel cofactor complex in the regulation of AR-dependent transcription. AR is an important regulatory factor in the development, differentiation, and maintenance of male reproductive functions, as well as in the regulation of other sexually dimorphic processes ranging from the development of neural tissues to the modulation of immune function. Thus, the p44-containing complex may play a pivotal role in these biological processes by modulating the transcriptional activity of AR.

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K. Hosohata and P. Li contributed equally to this work.

#### REFERENCES

1. Aarnisalo, P., J. J. Palvimo, and O. A. Janne. 1998. CREB-binding protein in androgen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA* **95**: 2122–2127.
2. Abdulkadir, S. A., J. A. Magee, T. J. Peters, Z. Kaleem, C. K. Naughton, P. A. Humphrey, and J. Milbrandt. 2002. Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. *Mol. Cell. Biol.* **22**:1495–1503.
3. Alen, P., F. Claessens, G. Verhoeven, W. Rombauts, and B. Peeters. 1999. The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. *Mol. Cell. Biol.* **19**:6085–6097.
4. Bentel, J. M., and W. D. Tilley. 1996. Androgen receptors in prostate cancer. *J. Endocrinol.* **151**:1–11.
5. Berger, S. L. 1999. Gene activation by histone and factor acetyltransferases. *Curr. Opin. Cell Biol.* **11**:336–341.
6. Berrevoets, C. A., P. Doesburg, K. Stekete, J. Trapman, and A. O. Brinkmann. 1998. Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor 2). *Mol. Endocrinol.* **12**:1172–1183.
7. Bhatia-Gaur, R., A. A. Donjacour, P. J. Scialolino, M. Kim, N. Desai, P. Young, C. R. Norton, T. Gridley, R. D. Cardiff, G. R. Cunha, C. Abate-Shen, and M. M. Shen. 1999. Roles for Nkx3.1 in prostate development and cancer. *Genes Dev.* **13**:966–977.
8. Bowen, C., L. Bubendorf, H. J. Voeller, R. Slack, N. Willi, G. Sauter, T. C. Gasser, P. Koivisto, E. E. Lack, J. Kononen, O. P. Kallioniemi, and E. P. Gelmann. 2000. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. *Cancer Res.* **60**:6111–6115.
9. Brinkmann, A. O., L. J. Blok, P. E. de Ruiter, P. Doesburg, K. Stekete, C. A. Berrevoets, and J. Trapman. 1999. Mechanisms of androgen receptor activation and function. *J. Steroid Biochem. Mol. Biol.* **69**:307–313.
10. Chen, H., R. J. Lin, W. Xie, D. Wilpitz, and R. M. Evans. 1999. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* **98**:675–686.
11. Choi, C. Y., Y. H. Kim, H. J. Kwon, and Y. Kim. 1999. The homeodomain



- protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J. Biol. Chem.* **274**:33194–33197.
12. Cillo, C., A. Faiella, M. Cantile, and E. Boncinelli. 1999. Homeobox genes and cancer. *Exp. Cell Res.* **248**:1–9.
  13. Claessens, F., P. Alen, A. Devos, B. Peeters, G. Verhoeven, and W. Rombauts. 1996. The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. *J. Biol. Chem.* **271**:19013–19016.
  14. Cleutjens, K. B., C. C. van Eekelen, H. A. van der Korput, A. O. Brinkmann, and J. Trapman. 1996. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J. Biol. Chem.* **271**:6379–6388.
  15. De Vos, P., F. Claessens, B. Peeters, W. Rombauts, W. Heyns, and G. Verhoeven. 1993. Interaction of androgen and glucocorticoid receptor DNA-binding domains with their response elements. *Mol. Cell. Endocrinol.* **90**: R11–R16.
  16. Fabbriozzi, E., S. El Messaoudi, J. Polanowska, C. Paul, J. R. Cook, J. H. Lee, V. Negre, M. Rousset, S. Pestka, A. Le Cam, and C. Sardet. 2002. Negative regulation of transcription by the type II arginine methyltransferase PRMT5. *EMBO Rep.* **3**:641–645.
  17. Friesen, W. J., S. Paushkin, A. Wyce, S. Massenet, G. S. Pesiridis, G. Van Duyn, J. Rappsilber, M. Mann, and G. Dreyfuss. 2001. The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. *Mol. Cell. Biol.* **21**:8289–8300.
  18. Friesen, W. J., A. Wyce, S. Paushkin, L. Abel, J. Rappsilber, M. Mann, and G. Dreyfuss. 2002. A novel WD repeat protein component of the methylosome binds Sm proteins. *J. Biol. Chem.* **277**:8243–8247.
  19. Gehring, W. J., M. Affolter, and T. Burglin. 1994. Homeodomain proteins. *Annu. Rev. Biochem.* **63**:487–526.
  20. Gelmann, E. P. 2002. Molecular biology of the androgen receptor. *J. Clin. Oncol.* **20**:3001–3015.
  21. Gu, W., and R. G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**:595–606.
  22. Heinlein, C. A., and C. Chang. 2002. Androgen receptor (AR) coregulators: an overview. *Endocr. Rev.* **23**:175–200.
  23. Ikonen, T., J. J. Palvimo, and O. A. Janne. 1997. Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. *J. Biol. Chem.* **272**:29821–29828.
  24. Janne, O. A., A. M. Moilanen, H. Poukka, N. Rouleau, U. Karvonen, N. Kotaja, M. Hakli, and J. J. Palvimo. 2000. Androgen-receptor-interacting nuclear proteins. *Biochem. Soc. Trans.* **28**:401–405.
  25. Kim, M. J., R. D. Cardiff, N. Desai, W. A. Banach-Petrosky, R. Parsons, M. M. Shen, and C. Abate-Shen. 2002. Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis. *Proc. Natl. Acad. Sci. USA* **99**:2884–2889.
  26. Kingston, R. E., and G. J. Narlikar. 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev.* **13**:2339–2352.
  27. Kundu, T. K., Z. Wang, and R. G. Roeder. 1999. Human TFIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. *Mol. Cell. Biol.* **19**:1605–1615.
  28. Li, P., X. Yu, K. Ge, J. Melamed, R. G. Roeder, and Z. Wang. 2002. Heterogeneous expression and functions of androgen receptor co-factors in primary prostate cancer. *Am. J. Pathol.* **161**:1467–1474.
  29. Ma, H., H. Hong, S. M. Huang, R. A. Irvine, P. Webb, P. J. Kushner, G. A. Coetzee, and M. R. Stallcup. 1999. Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. *Mol. Cell. Biol.* **19**:6164–6173.
  30. Malik, S., and R. G. Roeder. 2000. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. *Trends Biochem. Sci.* **25**:277–283.
  31. Martinez, E., V. B. Palhan, A. Tjernberg, E. S. Lyman, A. M. Gamper, T. K. Kundu, B. T. Chait, and R. G. Roeder. 2001. Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors in vivo. *Mol. Cell. Biol.* **21**:6782–6795.
  32. McKenna, N. J., and B. W. O'Malley. 2002. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**:465–474.
  33. McKenna, N. J., J. Xu, Z. Nawaz, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 1999. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J. Steroid Biochem. Mol. Biol.* **69**:3–12.
  34. Meister, G., C. Eggert, D. Buhler, H. Brahm, C. Kambach, and U. Fischer. 2001. Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Curr. Biol.* **11**:1990–1994.
  35. Meister, G., and U. Fischer. 2002. Assisted RNP assembly: SMN and PRMT5 complexes cooperate in the formation of spliceosomal snRNPs. *EMBO J.* **21**:5853–5863.
  36. Neely, K. E., and J. L. Workman. 2002. The complexity of chromatin remodeling and its links to cancer. *Biochim. Biophys. Acta* **1603**:19–29.
  37. Ogryzko, V. V., T. Kotani, X. Zhang, R. L. Schiltz, T. Howard, X. J. Yang, B. H. Howard, J. Qin, and Y. Nakatani. 1998. Histone-like TAFs within the PCAF histone acetylase complex. *Cell* **94**:35–44.
  38. Orlando, V. 2000. Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. *Trends Biochem. Sci.* **25**:99–104.
  39. Orlando, V., and R. Paro. 1993. Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. *Cell* **75**:1187–1198.
  40. Pollack, B. P., S. V. Kotenko, W. He, L. S. Izotova, B. L. Barnoski, and S. Pestka. 1999. The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. *J. Biol. Chem.* **274**:31531–31542.
  41. Prescott, J. L., L. Blok, and D. J. Tindall. 1998. Isolation and androgen regulation of the human homeobox cDNA, NKX3.1. *Prostate* **35**:71–80.
  42. Qi, C., J. Chang, Y. Zhu, A. V. Yeldandi, S. M. Rao, and Y. J. Zhu. 2002. Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. *J. Biol. Chem.* **277**:28624–28630.
  43. Roeder, R. G. 1998. Role of general and gene-specific cofactors in the regulation of eukaryotic transcription. *Cold Spring Harbor Symp. Quant. Biol.* **63**:201–218.
  44. Roeder, R. G. 1996. The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem. Sci.* **21**:327–335.
  45. Schneider, A., T. Brand, R. Zweigert, and H. Arnold. 2000. Targeted disruption of the Nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. *Mech. Dev.* **95**:163–174.
  46. Schuur, E. R., G. A. Henderson, L. A. Kmetec, J. D. Miller, H. G. Lamparski, and D. R. Henderson. 1996. Prostate-specific antigen expression is regulated by an upstream enhancer. *J. Biol. Chem.* **271**:7043–7051.
  47. Shen, E. C., M. F. Henry, V. H. Weiss, S. R. Valentini, P. A. Silver, and M. S. Lee. 1998. Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes Dev.* **12**:679–691.
  48. Stallcup, M. R., D. Chen, S. S. Koh, H. Ma, Y. H. Lee, H. Li, B. T. Schurter, and D. W. Aswad. 2000. Co-operation between protein-acetylating and protein-methylating co-activators in transcriptional activation. *Biochem. Soc. Trans.* **28**:415–418.
  49. Steadman, D. J., D. Giuffrida, and E. P. Gelmann. 2000. DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1. *Nucleic Acids Res.* **28**:2389–2395.
  50. Wang, H., Z. Q. Huang, L. Xia, Q. Feng, H. Erdjument-Bromage, B. D. Strahl, S. D. Briggs, C. D. Allis, J. Wong, P. Tempst, and Y. Zhang. 2001. Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* **293**:853–857.
  51. Wang, Q., D. Sharma, Y. Ren, and J. D. Fondell. 2002. A coregulatory role for the TRAP/Mediator complex in androgen receptor mediated gene expression. *J. Biol. Chem.* **277**:42852–42858.
  52. Wang, Z., and R. G. Roeder. 1998. DNA topoisomerase I and PC4 can interact with human TFIIC to promote both accurate termination and transcription reinitiation by RNA polymerase III. *Mol. Cell* **1**:749–757.
  53. Wang, Z., and R. G. Roeder. 1997. Three human RNA polymerase III-specific subunits form a subcomplex with a selective function in specific transcription initiation. *Genes Dev.* **11**:1315–1326.
  54. Xu, L., C. K. Glass, and M. G. Rosenfeld. 1999. Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.* **9**:140–147.
  55. Xu, W., H. Chen, K. Du, H. Asahara, M. Tini, B. M. Emerson, M. Montminy, and R. M. Evans. 2001. A transcriptional switch mediated by cofactor methylation. *Science* **294**:2507–2511.
  56. Yu, X., P. Li, R. G. Roeder, and Z. Wang. 2001. Inhibition of androgen receptor-mediated transcription by amino-terminal enhancer of split. *Mol. Cell. Biol.* **21**:4614–4625.
  57. Zhang, J., S. Zhang, P. E. Murtha, W. Zhu, S. S. Hou, and C. Y. Young. 1997. Identification of two novel cis-elements in the promoter of the prostate-specific antigen gene that are required to enhance androgen receptor-mediated transactivation. *Nucleic Acids Res.* **25**:3143–3150.
  58. Zhang, Y., and D. Reinberg. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev.* **15**:2343–2360.



## Modulation of Androgen Receptor-Dependent Transcription by Resveratrol and Genistein in Prostate Cancer Cells

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**BACKGROUND.** The androgen receptor (AR) is a ligand-activated transcription factor that mediates the biological responses of androgens in the prostate gland. This study focuses on the chemopreventive agents, resveratrol and genistein, on AR-mediated transcription in prostate cancer cells.

**RESULTS.** We found that resveratrol and genistein activated AR-driven gene expression at low concentrations, whereas they repressed the AR-dependent reporter gene activity at high concentrations. We determined that resveratrol and genistein induced AR-driven gene expression by activating the Raf–MEK–ERK kinase pathway. The ERK1 kinase phosphorylated the AR on multiple sites in vitro, but this phosphorylation event did not contribute to the resveratrol-induced AR transactivation.

**CONCLUSIONS.** In vitro and in vivo studies have indicated that resveratrol and genistein are promising chemopreventive agents. Given the clear evidence that AR pathways are involved in the development and progression of prostate cancer, these data showed that the ability to modulate AR function would contribute to the observed chemopreventive activity of resveratrol and genistein. *Prostate* 59: 214–225, 2004. © 2003 Wiley-Liss, Inc.

**KEY WORDS:** androgen receptor; resveratrol; genistein; chemoprevention; MAP kinase; prostate cancer

### INTRODUCTION

Androgens play an important role in the proliferation, maintenance, and function of the prostate gland [1,2]. Evidence shows that androgens are also involved in the development and progression of prostate cancer, which is the basis for treating non-organ-confined prostatic carcinoma with androgen ablation [3,4]. The androgen receptor (AR) mediates androgen functions and belongs to the nuclear receptor superfamily [5]. The AR, like other members of the super family, contains a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) with an associated activation function 2 (AF-2), and an N-terminal domain (NTD) containing the activation function 1 (AF-1) and the inhibition domain (ID) [6]. The AR binds to cognate androgen response elements (AREs) in target genes and, through its AF-1 and AF-2, interacts with various coactivators that facilitate transcription. Increasing numbers of cofactors have been implicated in the function of AR. These include p300/CBP, the

p160 family (SRC-1, TIF-2/GRIP-1, ACTR), TRAP/mediator complex, the ARA group, and others [7–9]. Little is known about how these cofactors function, and some of them show broader effects on basal transcription and other activators.

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Chemoprevention is a new strategy to prevent or slow prostate carcinogenesis [10]. Genistein, the most abundant phytochemical component of soy, has been implicated in the prevention of hormone-sensitive cancers including breast and prostate [11]. Prostate tumor incidence and growth have been reduced in animals exposed to a soy diet [12–15]. The growth and metastasis of transplantable tumors was inhibited in animals injected with purified genistein [16] and fed with genistein [17]. These studies support the epidemiological evidence that populations consuming diets rich in soy, have lower incidence of prostate cancer than those populations that consume diets with little or no soy [10,11]. Resveratrol, a bioflavonoid found in a multitude of dietary plants, including grapes and peanuts, was identified as a potential chemopreventive agent *in vitro* and in animal models [18]. Studies on resveratrol in rats indicate that even an average consumer of red wine, particularly over the long term, can absorb enough quantities of resveratrol to be beneficial to the health [19]. Subsequently, resveratrol has been shown to bind to the estrogen receptor (ER) [20] and is considered a phytoestrogen because of its potent estrogenic properties in MCF-7 human breast cancer cells [21], but additional studies conducted with these cells demonstrated that resveratrol has antagonistic activity in the presence of estradiol [21,22]. It has been shown that resveratrol inhibited the growth of androgen-responsive prostate cancer cells, in part because of the decreased expression of the AR [23]. The molecular mechanisms that contribute to the chemopreventive activities of genistein and resveratrol are still unclear.

Since the AR pathway plays a pivotal role in prostate cell growth, differentiation, and function, it is one of the possible sites of intervention in prostate cancer prevention efforts. In this study, we investigated the effects of resveratrol and genistein on AR-driven gene expression in the human prostate cancer cell lines LNCaP and PC3. We found that resveratrol and genistein enhanced AR transactivation at lower concentrations, and that they inhibited AR transcription at higher concentrations. Resveratrol and genistein activated AR function through the Raf–MEK–ERK signaling pathway.

## MATERIALS AND METHODS

### Plasmid Construction

We generated the various deletion mutations by doing polymerase chain reaction (PCR) on the corresponding nucleotide sequences of human AR cDNA. The PCR products were subcloned into pcDNA3.1 with the FLAG epitope at the N-terminal ends for a transient transfection assay. The AR point mutants were generated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and following the

manufacturer's instructions. The mutations were confirmed by DNA sequencing analysis. The expression vectors (NpT7-5 ERK1wt and pBB131 MEKKc) were kindly provided by Dr. Melanie Cobb (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX).

### Transient Transfection Assay

The PC3 and LNCaP cells were maintained in RPMI 1640 medium plus 10% fetal bovine serum. Approximately 24 hr before transfection,  $10^5$  cells were plated into each well of 24-well plates. The cells in each well were transfected with 10 ng of the vector expressing the wild-type or various mutated AR, 100 ng of the luciferase reporter plasmids, and 2.5 ng of the pR-LUC internal control plasmid. The total amount of DNA was adjusted to 300 ng with pcDNA3.1. The transfections were performed using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and were conducted in serum-free RPMI 1640 medium. The medium was changed 6 hr later, to phenol-free RPMI 1640 plus 10% charcoal-stripped fetal bovine serum or regular RPMI 1640 plus 10% fetal bovine serum. Various concentrations of resveratrol and genistein and 1 nM R1881 were added to the medium 24 hr after transfection. The cells were cultured for another 24 hr and then were harvested for the dual luciferase assay (Promega, Madison, WI).

### Immunoblots

Whole-cell lysates made from the transient transfected cells were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then proteins were transferred to a nitrocellulose membrane for Western blot analysis. The immunoblots were blocked for 30 min in 3% nonfat dry milk in TBST (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20). The blots were then incubated overnight with the anti-p44/p42 antibody and the anti-phospho-p44/p42 antibody (Cell Signaling Technology, Beverly, MA) or for 2 hr with the anti-FLAG or anti-actin antibodies (Sigma-Aldrich Corp., St. Louis, MO) or the anti-AR antibody. The blots were then washed with TBST three times and incubated for 1.5 hr with the second antibody (Amersham Biosciences Corp., Piscataway, NJ). Antibodies were diluted with 2% bovine serum albumin (BSA) in TBST. The protein bands were detected by an enhanced chemiluminescence kit (Amersham Biosciences).

### Protein Expression and Purification

The His<sub>6</sub>-tagged activated and inactivated ERK1 proteins were obtained by the expression of ERK1 with



and without the constitutively activated MEK1 mutant (MEK1-R4F) in *Escherichia coli* and purified through a  $\text{Ni}^{2+}$  nitrilotriacetic acid (NTA) agarose column (Qiagen, Valencia, CA) as described previously [24] with the following modifications. Expressions of the recombinant proteins were induced with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside at 30°C for 3 hr. The bacterial whole-cell lysates (from 1 L of the culture) were incubated with 1 ml of  $\text{Ni}^{2+}$ -NTA agarose. The beads were washed and then eluted with 0.2 M imidazole in BC100-0.1% Nonidet P-40 (NP40). The fractions containing the purified kinases were frozen in liquid nitrogen and stored at -80°C in small aliquots. The other His<sub>6</sub>-tagged and glutathione-S-transferase (GST)-tagged proteins were expressed similarly and purified through  $\text{Ni}^{2+}$ -NTA agarose or reduced glutathione-Sepharose (Amersham Biosciences), respectively.

### Protein Kinase Assay

The protein kinase assay was performed by incubating 25 ng of the recombinant activated ERK1 with 40 ng of recombinant factors in a 30  $\mu$ l volume of buffer containing 20 M HEPES (pH 7.3), 10 mM  $\text{MgCl}_2$ , 1 mM benzamidine, 1 mM 1,4-dithiothreitol, 100  $\mu$ M ATP, and 5  $\mu$ Ci  $\gamma$ -<sup>32</sup>P-ATP (Amersham Biosciences) for 30 min at 30°C. The reactions were stopped by adding 3.3  $\mu$ l of 100% trichloroacetic acid, and the precipitates were collected by centrifugation. The pellet was washed with acetone and dissolved in 20  $\mu$ l of 2 $\times$  SDS gel sample buffer and then was heated for 5 min at 95°C for analysis by SDS-PAGE followed by autoradiography.

## RESULTS

### Resveratrol and Genistein Modulate the AR-Driven Report Gene Activity

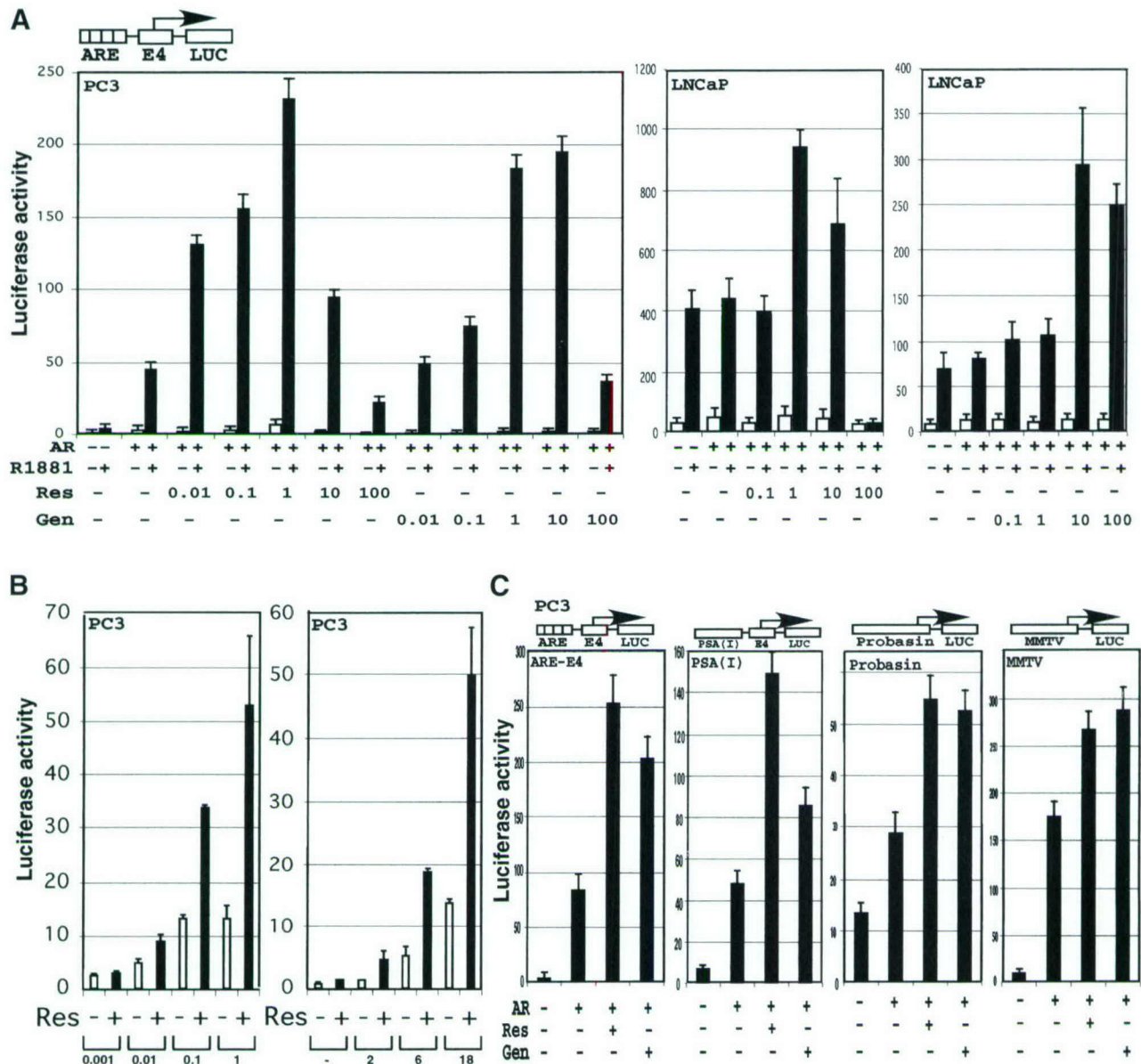
To determine if resveratrol and genistein could affect AR-driven gene expression, a transient transfection assay was performed in PC3 cells to study the transcriptional activities of AR in the presence of various concentrations of resveratrol and genistein. This cell line was selected because it does not express AR [25]. For initial tests, we used a synthetic hybrid promoter (pGL3-ARE-E4) containing four copies of the ARE derived from the prostate-specific antigen (PSA) promoter ahead of the adenovirus E4 core promoter and the luciferase gene [26]. As shown in Figure 1A, exogenous expression of AR activated the reporter gene about 10-fold in the presence of the synthetic androgen (R1881), and resveratrol and genistein further stimulated luciferase activity in a concentration-dependent manner (2.8–5.6 folds at 0.01–10  $\mu$ M of

resveratrol; 1.5–4 folds at 0.1–10  $\mu$ M of genistein), whereas 100  $\mu$ M resveratrol and genistein inhibited luciferase activity (twofold). Resveratrol and genistein at concentrations from 0.01 to 100  $\mu$ M had no obvious effect on luciferase activity in the absence of R1881, indicating their actions on AR-driven gene expression were dependent on the presence of androgen and, further, that resveratrol and genistein did not function as ligand agonists for AR. Structurally, resveratrol resembles the synthetic estrogen diethylstilbestrol, and resveratrol directly binds to the ER [20,21]. Resveratrol functions as an estrogen agonist in some human breast cancer cell lines [21] and as a mixed estrogen agonist/antagonist in others [20]. Neither resveratrol nor genistein functions as an agonist or antagonist for AR in either PC3 or LNCaP cells (see below). The fold-activation of AR transactivation (from 1.1- to 3.9-fold) by resveratrol was positively correlated with the concentrations of R1881 (from 0.001 to 1 nM) in PC3 cells (Fig. 1B, left panel).

Next, we performed the same assay with an AR-positive prostate cancer cell line (LNCaP). Because LNCaP cells contain high levels of endogenous AR [25], exogenous expression of AR did not further enhance AR-driven gene expression (Fig. 1A). Resveratrol (2.4–1.7 folds at 1–10  $\mu$ M) and genistein (4–3.3 folds at 10–100  $\mu$ M) stimulated luciferase activity. However, 100  $\mu$ M of resveratrol completely inhibited the luciferase activity (Fig. 1B), which was consistent with previous observations that resveratrol at concentrations of 100  $\mu$ M and higher strongly inhibited AR-driven gene expression in LNCaP cells [23]. Resveratrol at concentrations of 0.1  $\mu$ M or less and genistein at concentrations of 1  $\mu$ M or less had no obvious effect on luciferase activity in LNCaP cells. LNCaP cells express the mutant AR (T877A) and were responsive to resveratrol and genistein without exogenous expression of the wild-type AR (data not shown). When the increasing amounts (from 2 to 18 ng) of AR were used in the transient transfection assay in PC3 cells, the higher activation of the AR-driven luciferase reporter was observed but the fold-enhancement of AR transactivation (circa threefold) by resveratrol did not change (Fig. 1B, right panel). These results indicate that the different sensitivity of PC3 and LNCaP cells to resveratrol is not due to the different protein levels of AR in the transfected PC3 and LNCaP cells.

Because the consensus ARE (GGWACAnnn-TGTTCT) can function broadly with other steroid receptors [27], transcriptional specificity *in vivo* is likely conferred at least in part by promoter context and/or context-dependent functional interactions with other factors [28]. This necessitates analysis of natural AR-responsive promoters in order to more closely approximate the physiological situation. Thus, we





**Fig. 1.** Effects of resveratrol and genistein on AR-driven gene expression. **A: Left panel,** PC3 cells were transfected with 100 ng of the reporter PGL3-ARE-E4, 10 ng of pcDNA-AR, 2.5 ng of the internal control reporter pRL-LUC, and various amounts of pcDNA3.1 (300 ng of total DNA). The cells were treated 24 hr after transfection with ethanol (as a control) or with 1 nM R1881 and the indicated concentrations ( $\mu$ M) of resveratrol or genistein. The cells were then harvested 48 hr later, and luciferase activity was assayed by the Dual-luciferase Reporter Assay System (Promega). **B: Left panel:** Cells were treated 24 hr after transfection with ethanol (as a control) or 1  $\mu$ M of resveratrol and different concentrations (0.001–1 nM) of R1881 as indicated. **B: Right panel:** Cells were transfected with different amounts (0–6 ng) of pcDNA-AR as indicated and treated 24 hr after transfection with ethanol (as a control) or 1 nM R1881 and 1  $\mu$ M resveratrol. **A: Left panels:** LNCaP cells were transfected with 100 ng of the reporters PGL3-ARE-E4, 10 ng of pcDNA-AR, 2.5 ng of the internal control reporter pRL-LUC, and various amounts of pcDNA3.1 (300 ng of total DNA). The cells were treated 24 hr after transfection with ethanol (as a control) or with 1 nM R1881 and the indicated concentrations ( $\mu$ M) of resveratrol or genistein. The cells were then harvested 48 hr later, and luciferase activity was assayed. **C:** PC3 cells were transfected with 100 ng of the reporters PGL3-ARE-E4, PGL3-PSA(I), PGL3-Probasin, or PGL3-MMTV, 10 ng of pcDNA-AR, 2.5 ng of the internal control reporter pRL-LUC, and various amounts of pcDNA3.1 (300 ng of total DNA). Cells were treated 24 hr after transfection with ethanol (as a control) or 1 nM R1881 and 1  $\mu$ M resveratrol or 10  $\mu$ M genistein as indicated.



expanded the analysis to the PSA enhancer promoter [PSA(I)] [29,30], and the probasin promoter [31] as well as the mouse mammary tumor virus (MMTV) promoter [32]. Resveratrol (1  $\mu$ M) and genistein (10  $\mu$ M) also enhanced the luciferase activity from these natural promoters (Fig. 1C). However, folds (1.5–3) of the enhancement were slightly different among these AR-responsive promoters.

### Resveratrol and Genistein Enhanced AR-Driven Gene Activity Through the Raf-MEK-ERK Signaling Pathway

In order to know how resveratrol and genistein modulate AR-driven gene activity, we first examined if the AR protein level changed in cells treated with resveratrol and genistein. Western blot analysis demonstrated that the AR protein level did not change in LNCaP cells treated with resveratrol or genistein at concentrations of 10  $\mu$ M or less (Fig. 2A, top panel, lanes 3–5 and 9–11). However, at higher concentration (100  $\mu$ M), resveratrol dramatically decreased the AR protein level (Fig. 2A, top panel, lane 6). The same cell extracts (5  $\mu$ g) were analyzed by Western blot assay with anti-actin antibody as a control (Fig. 2A, bottom panel). Similar results were observed when PC3 cells transiently transfected with AR were treated with resveratrol and genistein (data not shown). These results imply that the higher concentration (100  $\mu$ M) of resveratrol inhibited the expression and/or stability of AR, leading to a decrease in AR-driven gene expression and other mechanism(s) may account for the resveratrol/genistein-induced AR-driven gene expression observed at the lower concentrations (1–10  $\mu$ M).

It is well documented that the AR signaling pathway can be activated by various growth factors through the signal transduction pathways [33]. In addition, it was demonstrated that low concentrations (1 pM to 10  $\mu$ M) of resveratrol could induce the phosphorylation of ERK1 and ERK2, while higher concentrations (50–100  $\mu$ M) inhibited this phosphorylation event in neuroblastoma cells [34]. Thus, we next examined whether resveratrol treatment could induce activation of the Raf-MEK-ERK signaling pathway in PC3 cells. Western blot analysis with the anti-phospho-p42/44 Mitogen-activated protein (MAP) kinase antibody, which specifically recognizes the phosphorylated (activated) forms of ERK1 and ERK2, revealed that upon treatment with resveratrol, ERK1 and ERK2 were phosphorylated (Fig. 2B, top panel, lane 2 vs. lane 1). However, the total protein levels of ERK1 and ERK2 did not change upon treatment with resveratrol (bottom panel, lane 2 vs. lane 1). The Raf-MEK-ERK pathway specific inhibitor PD098059 (Sigma-Aldrich), completely blocked activation of ERK1 and ERK2 by resver-

atrol (Fig. 2B, top panel, lane 3 vs. lane 2) and had no effect on the total protein levels of ERK1 and ERK2 (bottom panel, lane 3 vs. lane 2). As shown in Figure 2C, 20  $\mu$ M PD098059 completely blocked enhancement of the AR-driven gene expression induced by resveratrol (1  $\mu$ M) or genistein (10  $\mu$ M). These results imply that both resveratrol and genistein enhanced AR-driven gene expression through activation of the Raf-MEK-ERK signaling pathway. Ectopic expression of ERK1 in both PC3 and LNCaP cells increased the AR-driven gene expression to the similar extent as that induced by resveratrol or genistein (up to threefold) (Fig. 2D).

### ERK1 Phosphorylated AR, SRC-1, and p300 In Vitro

We next investigated whether ERK1 could directly phosphorylate AR or factors involved in AR-driven transcription. Multiple transcription factors seem to be involved in the control of AR-driven gene expression [7–9]. These include AR and its various cofactors. We expressed human AR [26], ARA70 (Fig. 3A, lane 2), SRC-1 (lanes 4–6), and p300 (lanes 7–9) in and purified from Sf9 cells. The method developed by Dr. Cobb's laboratory to reconstitute the MAP kinase phosphorylation cascades in bacteria was used to obtain the activated ERK1. In this system, the downstream kinase (ERK1) was coexpressed with the constitutively active upstream kinase (MEK1-R4F) to generate the activated (phosphorylated) MAP kinase. Using this system, we expressed and purified the inactivated (not-phosphorylated) ERK1 (Fig. 3A, lane 11) and the activated ERK1 (Fig. 3A, lane 12). The portion of the active ERK1 based on the shifted mobility was about 50% (Fig. 3A, lane 12). The activated ERK1 (p-ERK1) (Fig. 3A, lane 14), but not the inactivated ERK1 (lane 13), was recognized by the anti-phospho-ERK antibody, which specifically recognizes activated (phospho-T202/Y204) ERK in Western blot analysis. The in vitro protein kinase assay demonstrated that AR (Fig. 3B, lane 2), SRC-1 (lane 10), and p300 (lane 13), but not ARA70 (lanes 5–7), were phosphorylated by the activated ERK1 but not by the inactivated ERK1 (lanes 1, 9, and 12).

### ERK1 Phosphorylated the AR on Multiple Sites

In order to determine the sites of phosphorylation on the AR by ERK1, we subcloned the cDNAs encoding the NTD (amino acid residues 1–557), the DBD (amino acid residues 537–662), and the LDB (amino acid residues 663–917) into an expression vector (pET15d) and expressed them in bacteria as His<sub>6</sub>-fusion proteins (Fig. 4B). We then purified the proteins through the Ni<sup>2+</sup>-NTA agarose column and subjected them to an in vitro protein kinase assay with the purified recombinant ERK1. Figure 4A shows that the NTD (lanes 1–3)

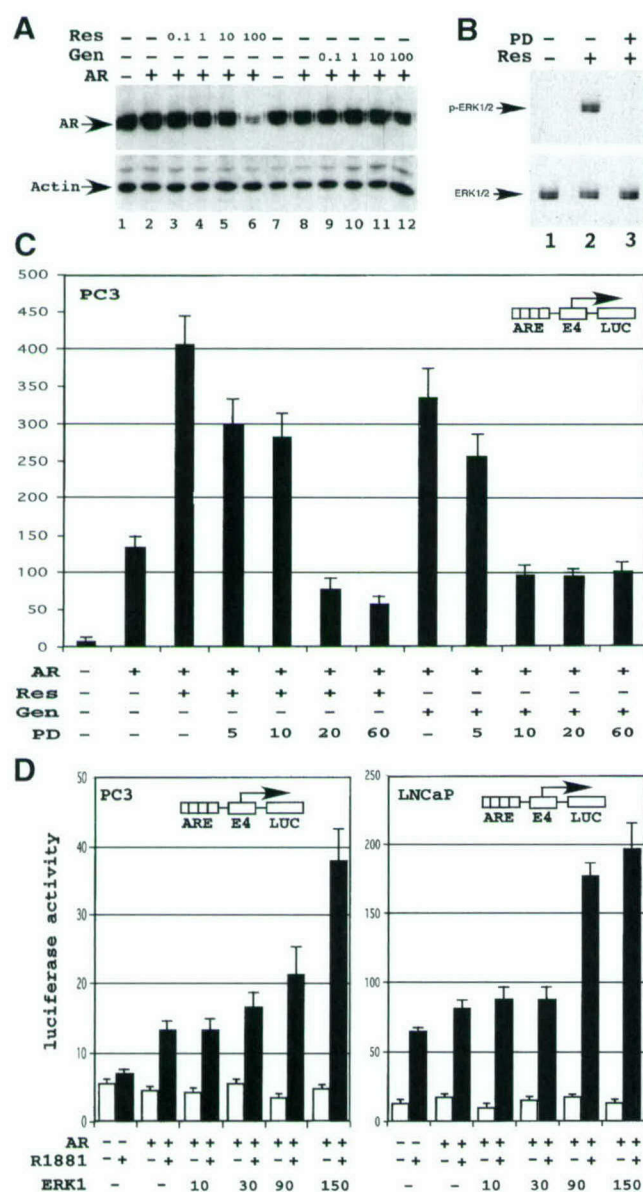


and the DBD (lanes 4–6), but not the LBD (lanes 7–9), were strongly phosphorylated by the activated ERK1. Two degraded bands (lane 3; indicated with stars at the left) from the NTD were also strongly phosphorylated by ERK1. As shown in Figure 4C, the fragments AR537–662 and AR558–662 (amino acid residues 537–662 and 558–662, respectively) were strongly phosphorylated by the activated ERK1 (lanes 2 and 11), whereas the fragment AR537–644 (amino acid residues 537–644) was only very weakly phosphorylated (lane 4), indicating that the region of the amino acid residues 645–662 (<sup>645</sup>ASSTTSPTTEETTQKLTVS<sup>662</sup>) contains the sites of phosphorylation by ERK1. Mutation of S650A in the AR558–662 fragment dramatically decreased the phosphorylation signal (lanes 11–13), indicating that S650 is a site phosphorylated by ERK1. The fragment

AR477–644 was strongly phosphorylated by ERK1 (lane 6), and a mutation at the amino acid residue serine<sup>515</sup> (S515A) almost completely abolished the phosphorylation signals (lane 8 vs. lane 6), whereas a mutation at the amino acid residue threonine<sup>541</sup> (T541A) did not dramatically affect the phosphorylation signals (lane 10 vs. lane 6). These results indicate that the amino acid residue S515 (<sup>511</sup>VPYSPSTCVK<sup>520</sup>) is another site of phosphorylation by ERK1. However, the mutation of S650A in AR did not affect the resveratrol-induced AR activation (data not shown).

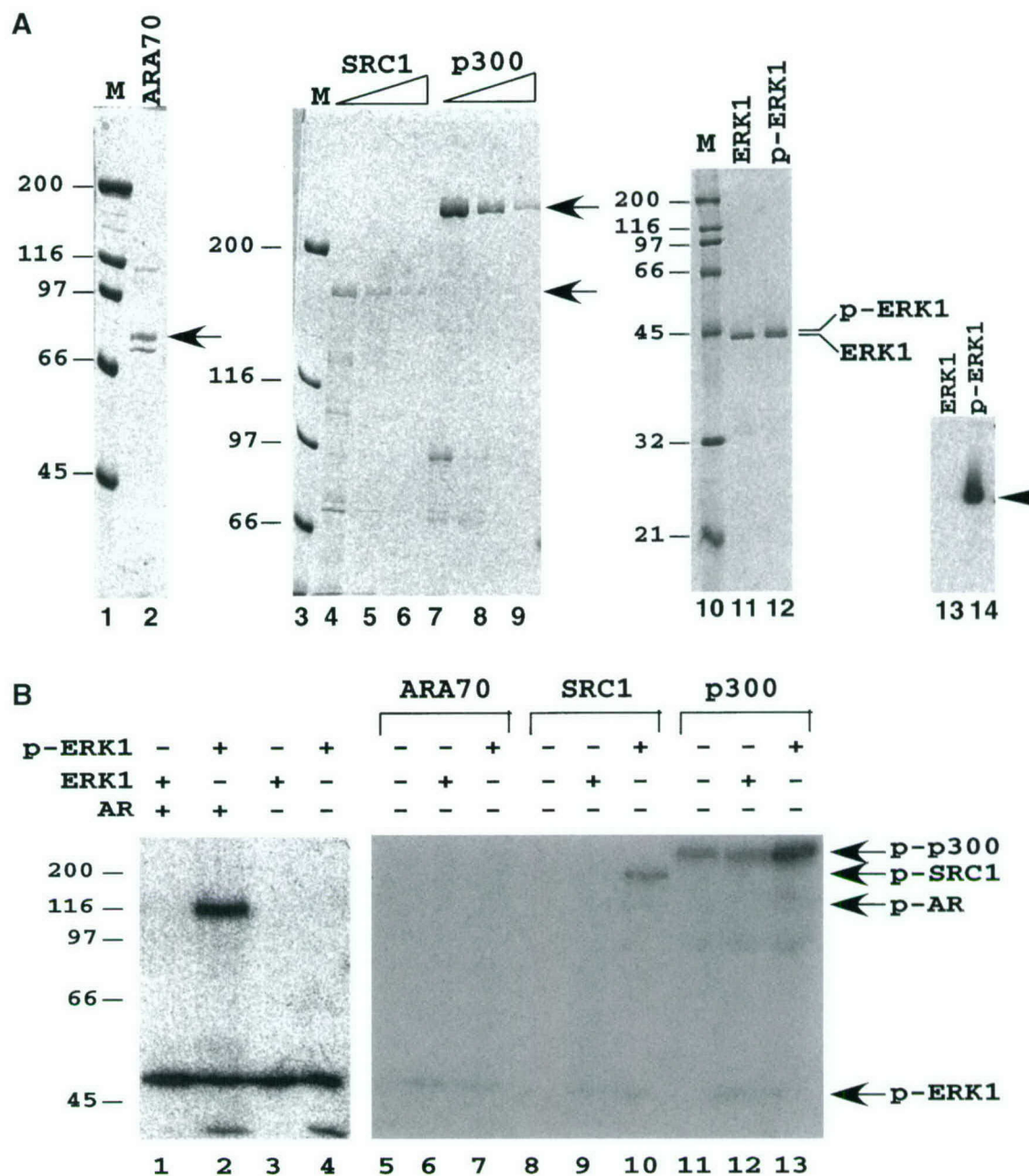
### Resveratrol Enhanced AR Transactivation by Modulating the NTD Function

Nuclear receptors contain two transcription activation functions, AF-1 in the NTD and AF-2 in the LBD. To analyze which domains account for the enhancement of AR transactivation, we generated different AR truncations and performed transient transfection experiments in the presence and absence of resveratrol. Transfection of the DBD/LBD (AR537–917) fragment produced only background levels of luciferase activity in the presence and absence of resveratrol (Fig. 5A). The NTD/DBD (AR1–643) fragment transactivated the luciferase gene to a greater extent than the full-length AR did, and the presence of resveratrol further enhanced



**Fig. 2.** Resveratrol and genistein activate AR-driven gene expression through the Raf-MEK-ERK signaling pathway. **A:** Western blot analysis of the whole cell extracts made from LNCaP cells treated without (lanes 1, 2, 7, and 8) or with various concentrations (μM) of resveratrol (lanes 3–6) or genistein (lanes 9–12) with anti-AR (top panel) and anti-actin (bottom panel) antibodies. Proteins were normalized by using the Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard and 5 μg proteins of each sample were loaded onto the SDS-polyacrylamide gel. **B:** Western blot analysis of extracts (40 μg) made from PC3 cells using the anti-Phospho-ERK antibody (top panel) or using the anti-ERK antibody (bottom panel). The cells were treated for 30 min with ethanol as a control (lane 1) or with 1 μM resveratrol (lane 2) or 1 μM resveratrol plus 20 μM PD098059 (lane 3). **C:** PD098059 inhibited activation of the AR-driven gene expression by resveratrol or genistein. PC3 cells were transfected with 100 ng of the reporter PGL3-ARE-E4, 10 ng of pcDNA-AR, 2.5 ng of pRL-LUC, and various amounts of pcDNA3.1. The cells were then treated 24 hr after transfection with ethanol as a control or 1 μM resveratrol or 10 μM genistein and the indicated concentrations (μM) of PD098059 24 hr after transfection. The cells were harvested 48 hr later for luciferase assay. **D:** ERK1 activates AR-driven gene expression. PC3 and LNCaP cells were transfected with 100 ng of the reporter PGL3-ARE-E4, 10 ng of pcDNA-AR, 2.5 ng of pRL-LUC, the indicated amounts (10–150 ng) of pcDNA-ERK1, and various amounts of pcDNA3.1. The cells were treated 24 hr after transfection with ethanol as control or 1 nM IRI881. The cells were harvested 48 hr later for the luciferase assay.



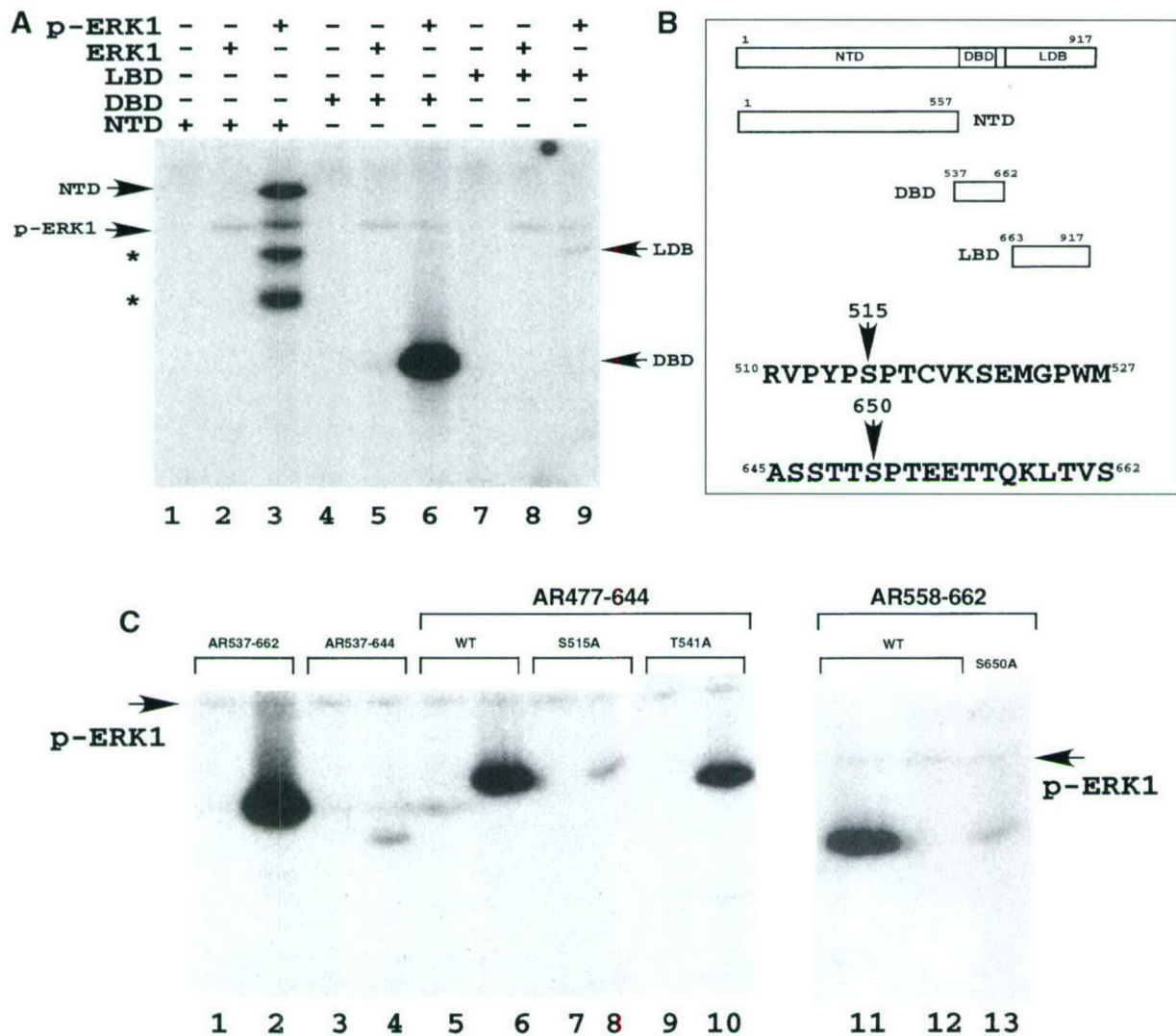


**Fig. 3.** ERK1 phosphorylates AR, SRC-1, and p300. **A:** SDS-PAGE analysis of the purified recombinant factors. The purified recombinant ARA70 (100 ng) (lane 2), SRC-1 (200, 100, and 50 ng) (lanes 4–6), p300 (400, 200, 100 ng) (lanes 7–9), and ERK1 (lanes 11 and 12) were loaded on SDS-gels and stained with Coomassie Blue R250. Lanes 1, 4, and 10 show molecular weight markers (Bio-Rad). Western blot analysis using the anti-phospho-ERK antibody of the purified recombinant inactivated ERK1 (lane 13) or the activated ERK1 (lane 14). **B:** ERK1 phosphorylates AR, SRC-1, and p300. Forty micrograms of the purified recombinant AR (lanes 1 and 2), ARA70 (lanes 6 and 7), SRC-1 (lanes 9 and 10), and p300 (lanes 12 and 13) were incubated with 25  $\mu$ g of the purified recombinant inactivated (lanes 1, 6, 9, 12) or the activated ERK1 (lanes 2, 7, 10, 13). The reaction mixtures were analyzed by 8% SDS-PAGE and the phosphorylated proteins were visualized by autoradiography. The bands corresponding to the phosphorylated proteins and to the autophosphorylated ERK1 are indicated by arrows at the right.

ed the reporter activity (2.2-fold). This effect was similar to that on the full-length AR (twofold) and was ligand-independent, demonstrating that the LDB is not involved in the enhancement of AR transactivation by resveratrol. Deletion of the N-terminal 206

amino acid residues did not affect hormone-independent activity or the response to resveratrol. However, when the N-terminal 267 amino acid residues were deleted, luciferase reporter activity dropped about 50%, and the response to resveratrol (2.4-fold) was not





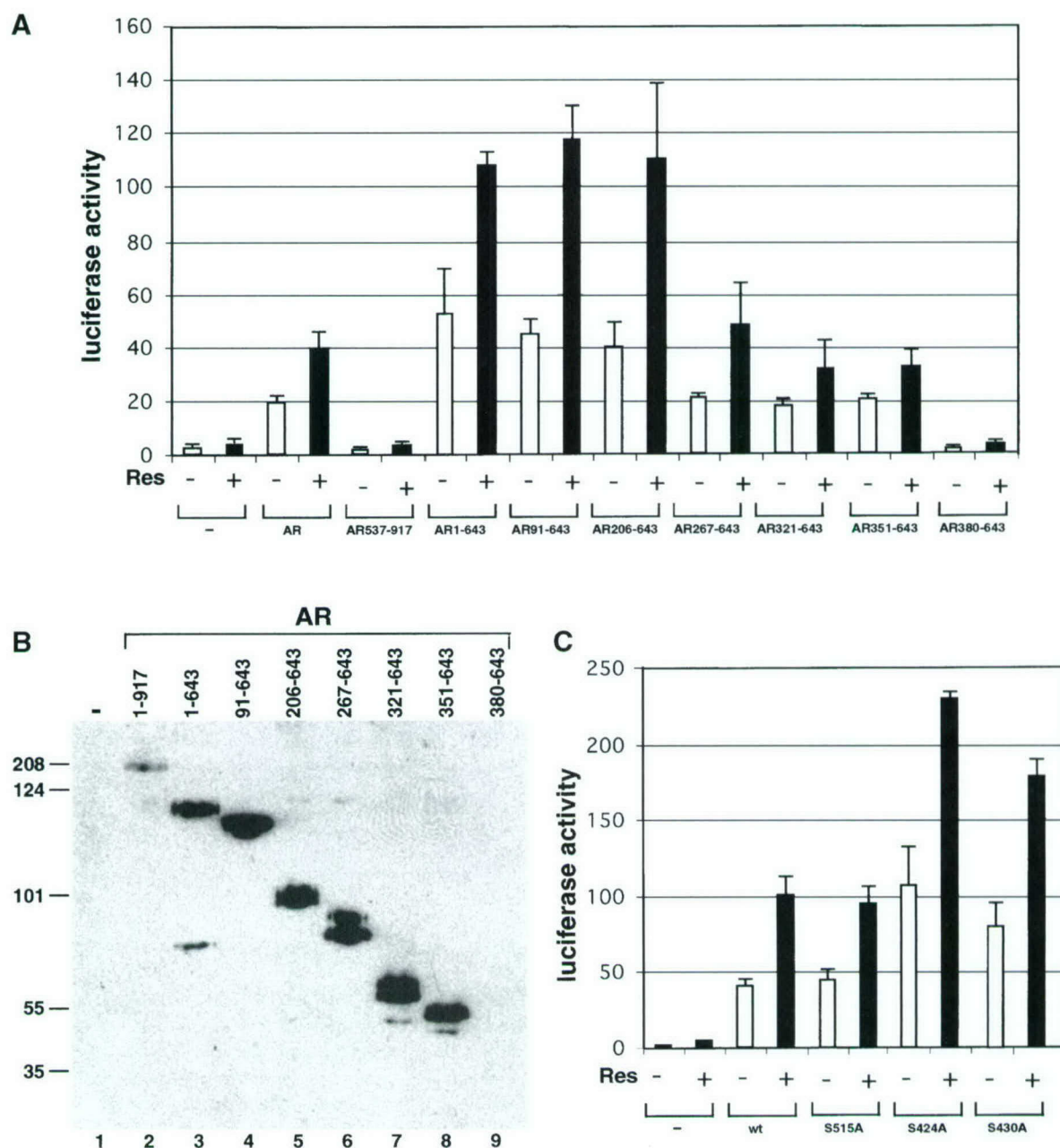
**Fig. 4.** ERK1 phosphorylates AR on multiple sites. **A:** ERK1 phosphorylates the NTD and DBD. The NTD (amino acid residues 1–557), DBD (amino acid residues 537–662), and LBD (amino acid residues 663–917) were expressed in bacteria and purified through the  $\text{Ni}^{2+}$ -NTA agarose column. One microgram of the purified proteins were subjected to an *in vitro* protein kinase assay without ERK1 (lanes 1, 4, 7), with 25  $\mu\text{g}$  of the inactivated ERK1 (lanes 2, 5, 8), or with 25  $\mu\text{g}$  of the activated ERK1 (lanes 3, 6, 9). **B:** Diagram of the domains of AR used for the protein kinase assay. **C:** ERK1 phosphorylates the amino acid residues S515 and S650. Various regions of the wild-type and mutant (S515A, T541A, S650A) AR fragments were expressed in bacteria via the pET15d vector and purified through the  $\text{Ni}^{2+}$ -NTA agarose column. The purified proteins (1  $\mu\text{g}$ ) were used for an *in vitro* protein kinase assay with 25  $\mu\text{g}$  of the inactivated ERK1 (lanes 1, 3, 5, 7, 9, 12) or 25  $\mu\text{g}$  of the activated ERK1 (lanes 2, 4, 6, 8, 10, 11, 13).

affected. Deletion of the amino acid residue preceding 351 had no additional effect on either transactivation (10-fold) or the response to resveratrol (1.8-fold). Deletion of the N-terminal 380 amino acid residues caused a loss of transactivation as well as a loss of response to resveratrol. Western blot analysis revealed that the protein levels of the AR truncations (Fig. 5B, lanes 3–8) were comparable and that they were much higher than those of the full-length AR (lane 2) in the transfected PC3 cells. However, the protein AR381–643 was undetectable by Western blot analysis with the anti-FLAG antibody (lane 9). This may cause the

undetectable activity of AR381–643 in transient transfection analysis (Fig. 5A). These results indicate that the NTD contains two activation domains, of which one contains amino acid residues 206–267 and the other contains amino acid residues 351–476. The region responsive to resveratrol is localized within amino acid residues 351–643.

Recently, we identified an ID that localizes in the NTD of the AR [6]. The ID interacts with the DBD and inhibits DBD–DNA interaction. ERK1 phosphorylated S515 (Fig. 4C), which localizes in the ID. However, a mutation of S515A had no effect on resveratrol-induced





**Fig. 5.** The NTD is involved in the resveratrol-induced activation of the AR. **A** and **C**: PC3 cells were transfected with 100 ng of the reporter PGL3-ARE-E4, 10 ng of vectors expressing the wild-type AR or mutated AR, 2.5 ng of the internal control reporter pRL-LUC, and various amounts of pcDNA3.1. The cells were then treated 24 hr after transfection with 1 nM RI88I and 10  $\mu$ M of resveratrol. The cells were harvested 48 hr later for the luciferase activity assay. **B**: Western blot analysis of the whole-cell lysates made from the transfected PC3 cells (panel A) with anti-FLAG monoclonal antibody.

AR transactivation (Fig. 5C), which indicated that the resveratrol did not affect AR transactivation through direct phosphorylation of the ID. Deletion of the ID (amino acid residues 477–556) did not affect the resveratrol-induced transactivation of the AR (data not shown). Thus, resveratrol enhanced AR transactivation not through the ID. Two amino acid residues

(S424 and S430) within amino acid residues 351–477 of AR are potential sites of phosphorylation by ERK kinase [37]. However, mutation of these sites did not affect the resveratrol-enhanced AR transactivation (Fig. 5C). Therefore, the enhancement of AR transactivation by resveratrol does not appear to be through phosphorylation of AR by the ERK kinase.



## DISCUSSION

In the current study, we found that resveratrol and genistein enhanced AR-dependent gene expression at lower concentrations but inhibited AR transactivation at higher concentrations. The resveratrol/genistein-induced activation of the AR occurred through activation of the Raf-MEK-ERK signaling pathway.

Chemoprevention is a new strategy in the fight against prostate cancer [10]. *In vitro* and *in vivo* studies have indicated that resveratrol and genistein are promising chemopreventive agents. Genistein may reduce the risk of cancer because of its antioxidant properties [38], as well as its ability to inhibit angiogenesis [39] and tyrosine kinase and [40] topoisomerase activity [41,42]. Genistein also enhance cell differentiation *in vitro* and in the mammary gland [43–46]. Given the clear evidence that AR pathways are involved in the development and progression of prostate cancer, the ability to modulate AR function may be another chemopreventive activity of resveratrol and genistein. The critical question is whether it is the activation or the inhibition of AR transactivation by genistein and resveratrol that prevents carcinogenesis. It is not clear what cellular levels of resveratrol and genistein *in vivo* could be reached through the diet. It has been reported that the average serum level of genistein in Japanese men is 0.6  $\mu\text{M}$  [47] and that the average resveratrol concentration in the plasma of rats was 1  $\mu\text{M}$  after the oral administration of red wine [48]. If one considers the fact that dietary phytochemicals are concentrated several-fold in the prostate gland and are components of prostatic secretion along with proteins such as PSA, 1–10  $\mu\text{M}$  concentrations of genistein or resveratrol in the prostate gland might be achievable through a diet rich in soy and/or grapes/red wine. If this is true, the activation of AR transactivation might play an important role in the chemopreventive activities of genistein and resveratrol. A high concentration of resveratrol (100  $\mu\text{M}$ ) decreased Chinese hamster ovary K1 cell proliferation in a manner dependent on the presence of ERs [20]. Similarly, a high concentration (>100  $\mu\text{M}$ ) of resveratrol inhibited prostate cancer cell (LNCaP) growth [23]. However, it is unknown, how resveratrol and genistein would affect prostate cell growth and differentiation at lower concentrations (1–10  $\mu\text{M}$ ).

The AR has strong constitutive AF-1 activity, and deletion of the LBD does not affect AR transactivation [49,50]. The AF-2 activity in AR is very weak since the deletion of the NTD results in a molecule that almost completely loses the ability to activate a reporter luciferase gene (Fig. 5A) [49]. This contrasts with what occurs in the ER, in which AR2 is the major activation domain [50,51]. The precise residues and mechanisms that contribute to the AF-1 activity of AR have not been

conclusively established. The entire NTD (residues 1–485) was necessary for full-length AR activity with the (GRE)<sub>2</sub>tkCAP reporter in HeLa cells, whereas a smaller part of the NTD (residues 360–528) was sufficient for constitutively active AR (residues 1–627) when the chimeric AR-GAL4 constructs were tested [50]. Recent investigations revealed that the AF-1 of the AR was a constituent of amino acid residues 360–494, which are mainly active in the absence of the LBD, and the amino acid residues 37–360, which are only active in the presence of the LBD [49]. Our results demonstrated that there are two distinct activation domains (AD1 and AD2) in the NTD of AR in PC3 cells. The AD1 contains amino acid residues 206–267, and AD2 spans amino acid residues 351–447. AD1 and AD2 show additive effects on the transactivation of AR. These data indicate that the size and location of the activation domains in the human AR are variable, depending on the promoter context, the presence or absence of the LBD, and cell types.

The Raf-MEK-ERK signal transduction cascade plays a critical role in the regulation of cell growth and differentiation [35,36]. The signaling pathway is primarily activated in response to various intracellular factors that are able to initiate intracellular signaling, which ultimately causes altered regulation of gene expression. Activation of the Raf-MEK-ERK pathway occurs through phosphorylation of threonine (T202) and tyrosine (Y204) in ERK by MEK, leading to the phosphorylation of transcription factors that directly influence specific gene expression through interactions with upstream regulatory elements. Phosphorylation of some transcription factors plays a critical role in the expression of their target genes. Upon phosphorylation, some transcription factors translocate from the cytosol to the nucleus to activate transcription of their genes, and others, originating in the nucleus, localize to their sequences on the DNA template in order to enhance transcription efficiency [52]. The transcription factors CREB, c-Myc, Ets, AP-1, and NF- $\kappa$ B are targets of the Raf-MEK-ERK cascade [36]. For example, the activated ERK phosphorylated c-Myc on S62, thus stimulating activity of the transactivation domain of c-Myc [52]. The human AR can be activated in the absence of androgens by growth factors and molecules/compounds that modulate protein kinase pathways [33]. It has been suggested that ligand-independent activation of the AR is one of possible mechanisms underlying androgen-independent prostate cancer [53,54]. The events involved in ligand-independent activation of the AR by protein kinase pathways are unknown, but it has been suggested that it involves phosphorylation of the AR itself and/or AR cofactors. Interleukin-6 (IL-6) can activate AR in the absence of androgens by activation of the MAP kinase



pathway in LNCaP cells [55]. Phosphorylation of SRC-1 by MAPK was required for optical ligand-independent activation of the AR by IL-6. Our results indicate that the activation of the MAP kinase pathway (Raf-MERK-ERK) by resveratrol and genistein is responsible for the enhancement of AR transactivation. This enhancement was dependent on androgens and apparently not the phosphorylation of the AR itself. Our in vitro study demonstrated that ERK1 directly phosphorylated SRC1 and p300, which function as AR cofactors [7–9]. The future study will be to investigate whether the phosphorylation event of SRC1 and p300 by ERK1 could lead to the transactivation of AR in prostate cancer cells.

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### REFERENCES

1. Frick J, Aulitzky W. Physiology of the prostate. *Infect* 1991; 19(Suppl 3):S115–S118.
2. Davies P, Eaton CL. Regulation of prostate growth. *J Endocrinol* 1991;131(1):5–17.
3. Bentel JM, Tilley WD. Androgen receptors in prostate cancer. *J Endocrinol* 1996;151(1):1–11.
4. Jenster G. The role of the androgen receptor in the development and progression of prostate cancer. *Semin Oncol* 1999;26(4): 407–421.
5. Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Stekete K, Berrevoets CA, Trapman J. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* 1999; 69(1–6):307–313.
6. Liu GZ, Wang H, Wang Z. Identification of a highly conserved domain in the androgen receptor that suppresses the DNA-binding domain–DNA interactions. *J Biol Chem* 2003.
7. Heinlein CA, Chang C. Androgen receptor (ar) coregulators: An overview. *Endocr Rev* 2002;23(2):175–200.
8. Janne OA, Moilanen AM, Poukka H, Rouleau N, Karvonen U, Kotaja N, Hakli M, Palvimo JJ. Androgen-receptor-interacting nuclear proteins. *Biochem Soc Trans* 2000;28(4):401–405.
9. Gelmann EP. Molecular biology of the androgen receptor. *J Clin Oncol* 2002;20(13):3001–3015.
10. Denis L, Morton MS, Griffiths K. Diet and its preventive role in prostatic disease. *Eur Urol* 1999;35(5–6):377–387.
11. Adlercreutz H. Phytoestrogens: Epidemiology and a possible role in cancer protection. *Environ Health Perspect* 1995; 103(Suppl 7):103–112.
12. Pollard M, Luckert PH. Influence of isoflavones in soy protein isolates on development of induced prostate-related cancers in 1-w rats. *Nutr Cancer* 1997;28(1):41–45.
13. Pollard M. Prevention of prostate-related cancers in Lobund-Wistar rats. *Prostate* 1999;39(4):305–309.
14. Landstrom M, Zhang JX, Hallmans G, Aman P, Bergh A, Damber JE, Mazur W, Wahala K, Adlercreutz H. Inhibitory effects of soy and rye diets on the development of dunning r3327 prostate adenocarcinoma in rats. *Prostate* 1998;36(3):151–161.
15. Onozawa M, Kawamori T, Baba M, Fukuda K, Toda T, Sato H, Ohtani M, Akaza H, Sugimura T, Wakabayashi K. Effects of a soybean isoflavone mixture on carcinogenesis in prostate and seminal vesicles of f344 rats. *Jpn J Cancer Res* 1999;90(4): 393–398.
16. Schleicher RL, Lamartiniere CA, Zheng M, Zhang M. The inhibitory effect of genistein on the growth and metastasis of a transplantable rat accessory sex gland carcinoma. *Cancer Lett* 1999;136(2):195–201.
17. Mentor-Marcel R, Lamartiniere CA, Eltoum IE, Greenberg NM, Elgavish A. Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (tramp). *Cancer Res* 2001;61(18):6777–6782.
18. Jang M, Pezzuto JM. Cancer chemopreventive activity of resveratrol. *Drugs Exp Clin Res* 1999;25(2–3):65–77.
19. Bertelli AA, Giovannini L, Stradi R, Urien S, Tillement JP, Bertelli A. Evaluation of kinetic parameters of natural phytoalexin in resveratrol orally administered in wine to rats. *Drugs Exp Clin Res* 1998;24(1):51–55.
20. Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. *Endocrinology* 2000;141(10):3657–3667.
21. Gehm BD, McAndrews JM, Chien PY, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc Natl Acad Sci USA* 1997;94(25): 14138–14143.
22. Mgbonyebi OP, Russo J, Russo IH. Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. *Int J Oncol* 1998;12(4):865–869.
23. Mitchell SH, Zhu W, Young CY. Resveratrol inhibits the expression and function of the androgen receptor in Lncap prostate cancer cells. *Cancer Res* 1999;59(23):5892–5895.
24. Khokhlatchev A, Xu S, English J, Wu P, Schaefer E, Cobb MH. Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria. Efficient synthesis of active protein kinases. *J Biol Chem* 1997;272(17):11057–11062.
25. Tilley WD, Wilson CM, Marcelli M, McPhaul MJ. Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res* 1990;50(17):5382–5386.
26. Yu X, Li P, Roeder RG, Wang Z. Inhibition of androgen receptor-mediated transcription by amino-terminal enhancer of split. *Mol Cell Biol* 2001;21(14):4614–4625.
27. Roche PJ, Hoare SA, Parker MG. A consensus DNA-binding site for the androgen receptor. *Mol Endocrinol* 1992;6(12):2229–2235.
28. Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, Rombauts W. Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *J Steroid Biochem Mol Biol* 2001;76(1–5):23–30.
29. Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J Biol Chem* 1996;271(11):6379–6388.
30. Schuur ER, Henderson GA, Kmetec LA, Miller JD, Lamparski HG, Henderson DR. Prostate-specific antigen expression is regulated by an upstream enhancer. *J Biol Chem* 1996;271(12): 7043–7051.
31. Reid KJ, Hendy SC, Saito J, Sorensen P, Nelson CC. Two classes of androgen receptor elements mediate cooperativity through allosteric interactions. *J Biol Chem* 2001;276(4):2943–2952.
32. Van Dijck P, Winderickx J, Heyns W, Verhoeven G. Binding of androgen-receptor complexes to alpha 2u-globulin genes and to



- the long terminal repeat of mouse mammary tumor virus. *Mol Cell Endocrinol* 1989;64(2):195–204.
33. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* 1994;54(20):5474–5478.
34. Miloso M, Bertelli AA, Nicolini G, Tredici G. Resveratrol-induced activation of the mitogen-activated protein kinases, erk1 and erk2, in human neuroblastoma sh-sy5y cells. *Neurosci Lett* 1999;264(1-3):141–144.
35. Hazzalin CA, Mahadevan LC. Mapk-regulated transcription: A continuously variable gene switch? *Nat Rev Mol Cell Biol* 2002;3(1):30–40.
36. Lee JT Jr, McCubrey JA. The raf/mek/erk signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* 2002;16(4):486–507.
37. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlege RE, Shabanowitz J, Hunt DF, Weber MJ. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J Biol Chem* 2002;277(32):29304–29314.
38. Wei H, Bowen R, Cai Q, Barnes S, Wang Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. *Proc Soc Exp Biol Med* 1995;208(1):124–130.
39. Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc Natl Acad Sci USA* 1993;90(7):2690–2694.
40. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987;262(12):5592–5595.
41. Okura A, Arakawa H, Oka H, Yoshinari T, Monden Y. Effect of genistein on topoisomerase activity and on the growth of [val 12]ha-ras-transformed nih 3t3 cells. *Biochem Biophys Res Commun* 1988;157(1):183–189.
42. Kaufmann WK. Human topoisomerase II function, tyrosine phosphorylation, and cell cycle checkpoints. *Proc Soc Exp Biol Med* 1998;217(3):327–334.
43. Constantinou A, Huberman E. Genistein as an inducer of tumor cell differentiation: Possible mechanisms of action. *Proc Soc Exp Biol Med* 1995;208(1):109–115.
44. Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis* 1996;17(7):1451–1457.
45. Fritz WA, Coward L, Wang J, Lamartiniere CA. Dietary genistein: Perinatal mammary cancer prevention, bioavailability, and toxicity testing in the rat. *Carcinogenesis* 1998;19(12):2151–2158.
46. Lamartiniere CA. Protection against breast cancer with genistein: A component of soy. *Am J Clin Nutr* 2000;71(6 Suppl):1705S–1707S discussion 1708S–1709S.
47. Pumford SL, Morton MM, Turkes A, Griffiths K. Determination of the isoflavonoids genistein and daidzein in biological samples by gas chromatography–mass spectrometry. *Ann Clin Biochem* 2002;39(Pt. 3):281–292.
48. Bertelli AA, Giovannini L, Stradi R, Urien S, Tillement JP, Bertelli A. Kinetics of *trans*- and *cis*-resveratrol (3,4',5-trihydroxystilbene) after red wine oral administration in rats. *Int J Clin Pharmacol Res* 1996;16(4–5):77–81.
49. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. The af1 and af2 domains of the androgen receptor interact with distinct regions of src1. *Mol Cell Biol* 1999;19(12):8383–8392.
50. Jenster G, van der Korput HA, Trapman J, Brinkmann AO. Identification of two transcription activation units in the n-terminal domain of the human androgen receptor. *J Biol Chem* 1995;270(13):7341–7346.
51. Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P. The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 1989;59(3):477–487.
52. Seth A, Alvarez E, Gupta S, Davis RJ. A phosphorylation site located in the nh2-terminal domain of c-myc increases transactivation of gene expression. *J Biol Chem* 1991;266(35):23521–23524.
53. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the her-2/neu tyrosine kinase. *Nat Med* 1999;5(3):280–285.
54. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From her2/neu signal cascade to androgen receptor and its coactivators: A novel pathway by induction of androgen target genes through map kinase in prostate cancer cells. *Proc Natl Acad Sci USA* 1999;96(10):5458–5463.
55. Ueda T, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. *J Biol Chem* 2002;277(41):38087–38094.